

A zebrafish model system

Studying uptake of particles and bacterial infections through the zebrafish intestine

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October 2015

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2015

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Trykk: Reprosentralen, Universitetet i Oslo

Acknowledgment

First, I would like to thank my supervisors Gareth Griffiths and Erling Olaf Koppang, for letting me start this project, and for their enthusiasm and guidance. I would also like to give a big thanks to my co-supervisor Martin Speth, for excellent feedback and lending me his time when I needed help. I would also like to thank Federico for help with PLGA particles, Urska Repnik for help with lectins and bacterial growth, and Jon Hildal for continuing with the project. I would need to thank the rest of the Griffith group, for their help and support; Carina Beatrice Vibe, Tone Lian, Håkon Høgseth, Patrick Lie Johansen, Lilia Ulanova, Shala Bagherifam, Gerbrand Koster, and Bård Mathiesen.

In addition, I would like to thank Guro Løkka, Lars Austbø and Alf Seljenes Dalum, in Koppangs group at NMBU, for giving me feedback on the project. Leidy Ximena Lagos Rojas for help with the flow cytometry and Julia Isabel Tandberg for help with growing *F.n.o* bacteria.

At last, I would like to thank my family and boyfriend, Pål Gunnar, for always believing in me and supporting me in everything I do.

Trondheim, October 2015

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Abstract

In aquaculture today, vaccination of farmed fish is successfully providing protection against many of the most common pathogens. However, while vaccines are most commonly given as a single injection, this is not the ideal way of administration, as it is stressful for the fish and labour intensive and costly. A more desirable way of administration is by the oral route, where vaccine-formulations can be incorporated into the fish feed and given repeatedly. Encapsulation strategies such as nanoparticles, that protect antigens and give a long and sustained release, have shown to be a promising strategy for oral vaccinations. However, little is known about the mechanisms involved in uptake and trans-epithelial transport of particles in the nano/micrometre range in the fish intestinal system. To date, there are only a few studies addressing the effect of different characteristics of particles on uptake, transport and immune stimulation capability in fish. Here we report an adult zebrafish model system to study the uptake of different types of particles in the fish intestine and their interactions with epithelial cells and the mucosal immune system.

We have used a protocol for oral intubation, which enables us to deliver fluorescent particles and bacteria directly into the adult zebrafish intestine. By histochemical methods and confocal laser microscopy, we are able to follow the uptake, transport and distribution of particles in the intestine and other organs over time. In a second line of experiments, we studied the ability of a well-known bacterial fish pathogen (*M. marinum*) to infect via the gastro-intestinal system in our model system. Oral intubation of adult zebrafish is therefore a powerful model system to study both the uptake and transport of particles via the gastro-intestinal tract and the interaction of pathogenic bacteria with the fish intestinal epithelia.

Selected abbreviations

Chi/pIC	Chitosan poly (I:C)
CLPs	Carboxylated latex particles
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal tract
GIALT	Gill-associated lymphoid tissue
HE	Hematoxylin and Eosin
IEL	Intraepithelial lymphocytes
LP	Lamina propria
MALT	Mucosal associated lymphoid tissue
M cells	Microfold cells
<i>M.m</i>	<i>Mycobacterium marinum</i>
NPs	Nanoparticles
o/n	Over night
PBST	PBS tween-20
PBSX	PBS Triton-X
PFA	Paraformaldehyde
PLGA	Poly lactide co-glycolic acid
Poly (I:C)	Polyinosinic polycytidylic acid
PVA	Polyvinyl alcohol

PP	Peyer's patches
RT	Room temperature
SALT	Skin-associated lymphoid tissue
SV	Supranuclear vacuoles
TB	Tuberculosis
TEM	Transmission electron microscopy
UEA-I	Ulex europaeus agglutinin
WGA	Wheat germ agglutinin

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1 Introduction

1.1 The mucosal barrier

In biology, a pathogen is a term used to describe an infectious agent, such as a virus, bacterium, prion, fungus, viroid or parasite that causes diseases in the host. Pathogens are living organisms specialized to infect the host's body, where they can reproduce and cause irritation and damage that may give rise to symptoms of infection. To infect, pathogens take advantage of weaknesses in the host's barriers to the external environment. However, vertebrates have developed a defence system against invasion from pathogens, the immune system. This active system recognizes, fights, and contains invading pathogens when detected. The evolutionary race between the host's immune system and the pathogen to evolve fastest and out-manoeuvre each other, has resulted in complex strategies to recognize and fight pathogens on one side, and to evade this defence on the other side

In vertebrates, 90 % of all infections occur via mucosal surfaces [2]. Mucosal surfaces are lined with epithelial cells covered in mucus, which creates an interface between the body and the external environment, such as in the eyes, and the respiratory, gastrointestinal, and genital tract in mammals. Mucosal surfaces are often involved in transmembrane transport, like nutrient uptake in the intestine or gas exchange in the lungs, and therefore they are required to be a selectively permeable barrier. Often, this permeability is targeted by pathogens using a wide variety of strategies.

1.1.1 Mucosal immune system

In vertebrates, the immune system has classically been divided into two components, the innate and the adaptive immune system. The innate immune system is the first line of defense and includes both physical barriers, like the mucus layer, and non-specific humoral and cellular responses. The adaptive immune system induces specific humoral and cellular responses. It is characterized by recognition of specific antigens and formation of immunological memory that enables a stronger and faster secondary immune response to a repeated infection. The innate immune system comprises of natural killer cells (NKC) and professional antigen presenting cells (APCs), such as dendritic cells (DC) and macrophages (Mφs), and the complement system. The adaptive system comprises mainly of B and T

lymphocytes [3]. However, the mucosal immune system is a complex network of both adaptive and innate immune components.

The mucus layer is made up of gel-forming mucin and the glycocalyx, which cover the epithelium and is part of the innate immune system that acts as a mechanical barrier against pathogens. In addition, various antimicrobial peptides produced by the epithelium are secreted into the mucus and may limit bacterial growth [4]. Cells of the innate immune system furthermore release pro- and anti-inflammatory cytokines as response to pathogen infection, which subsequently attract, activate and regulate the immune response of the adaptive immune system.

In mammals, the lymphatic system, which includes lymphatic organs, a conducting network of lymphatic vessels and the circulating lymph fluid, is an important part of the immune system. In the lymph, leukocytes and other lymphocytes are transported from and to areas of inflammation. Activated dendritic cells and macrophages from infection sites migrate to associated lymphatic organs (lymph nodes) where they present antigens from pathogens to naïve lymphocytes, which finally results in priming and activation of antigen-specific B- and T- cells. In many mucosal epithelia, specialized lymphatic tissue termed mucosal associated lymphoid tissue (MALT), have corresponding functions to the lymph nodes. MALT are more diffuse structures of lymphoid tissue and are found in the gastrointestinal (GI) tract, skin, lungs, eyes, breasts, and salivary glands. In the gut, MALT is further distinguished as the gut associated lymphoid tissue (GALT) with structures like Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) [5].

The mucosal immune system in the gut, can be divided into two sites: the induction site with the GALT, and the effector site with the lamina propria (LA) and the intraepithelial lymphocytes (IEL) compartment [6]. Antigen specific mucosal immunity is dependent on the uptake of antigens and whole bacteria microbes from the intestinal lumen and them being presented to B- and T- cells in lymph nodes. In mammals, Microfold (M-) cells located above the PP are essential for antigen processing at the induction site [7]. These cells can actively transport antigens across the epithelia to APCs, like DC, which then migrate to the mesenteric lymph node. DCs can also extend processes in between the epithelial cells and sample the intestinal lumen for exogenous antigens directly [8]. In the lymph node, DCs prime naïve T cells, which in turn facilitates immunoglobulin (Ig) class switch recombination and somatic hyper-mutations in B cells. Ig's are produced by plasma cells and plasmablasts (mature B

cells), and are secreted into body fluids as antibodies or are present on the cell membrane as B cells receptors (BCR). In mammals, five Ig isotypes are commonly found: IgA, IgG, IgD, IgE and IgM, where IgA is most commonly secreted in the intestinal mucosa.

In the lymph node, B cells mature into IgA⁺ plasmablasts, home to the mucosal effector site and mature further to IgA⁺ plasma cells that secrete dimeric IgA. Dimeric IgA forms a complex with the polymeric immunoglobulin receptor and is transcytosed across the intestinal lumen. At the luminal site, part of the receptor is cleaved off and become secretory IgA (SIgA). SIgA protect through preventing antigens to enter the body (immune exclusion), intracellular neutralization of the antigen, and antigen excretion (fig.1) [9].

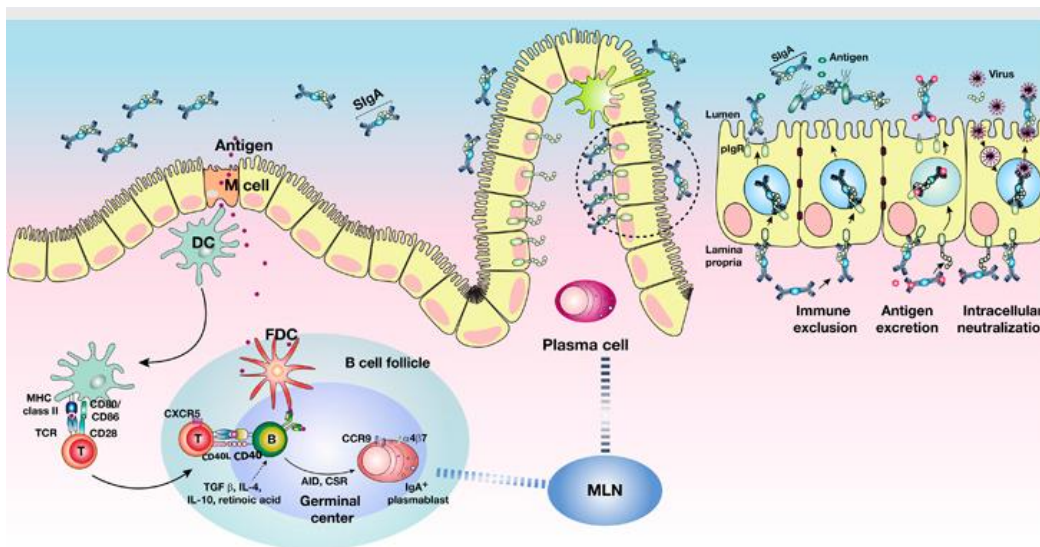


Figure 1: Schematic overview of T cell-dependent induction of IgA production at mucosal sites (intestine). In PP, antigen are transcytosed by M cells in the epithelium, and presented to DC (APCs) underneath. DC loaded with antigen migrate to the mesenteric lymph nodes, prime and induce maturation of naïve T cells into effector T cells. CD4⁺ T cells expressing CD40 ligand induce IgA class-switch recombination through interaction with B cells expressing CD40 receptor. IgA⁺ plasmablasts home to the mucosal effector site, where they secrete dimeric IgA, which interacts with the polymeric Ig receptor and is finally transcytosed across to the intestinal lumen as SIgA. SIgA protects the host by immune exclusion, antigen excretion and intracellular neutralization. Figure taken from Kim and Jang [2].

1.2 Mucosal immunity in the teleost intestine

Teleost fish include most species of fish, such as zebrafish, salmon, and cod [10] and are part of the superclass Osteichthyes (bony fish). In fish, the mucosal surfaces are found in the eyes, skin, gills, and the reproductive and gastro-intestinal tract. The fish intestine, as in all vertebrates, is a multifunctional organ, which carries out many important and diverse physiological functions. Next to nutrient uptake and waste excretion, defence against pathogens is one of the most important functions.

The lymphoid system in fish are much more diffuse than in mammals, as they lack bone marrow, lymph nodes and essential GALT structures like Peyer's patches. In addition, other essential parts of the mucosal immune system like M-cells, IgA, and J-chain (binding polymeric immunoglobulins secreted into the lumen) have so far not been shown in teleost fish [6]. Even though important mucosal immune components are not found in fish, several studies detect specific antibodies in the mucosal secretions after intestinal [11-14] or immersion [15-17] immunization in a variety of fish species, while they were hardly detected after systemic immunization [18]. Teleost fish also have a highly developed complement system, an important component of the innate immune system, as shown in several teleost species [19, 20].

1.2.1 Mucosal immune components in teleost fish

The earliest recognizable adaptive immune system arose in teleost fish 500 million years ago [21]. It comprises of B- and T- lymphocytes with antigen receptors, major histocompatibility complex (MHC), and immunological memory [22]. Immunoglobulin positive (Ig+) cells are described in the intestinal mucosa of several teleost species, including sea bass [23, 24], cyprinids [25], and salmonids [26]. There are three major Ig isotypes reported in teleost fish: IgM, IgD, and IgT [27, 28]. IgM is the main Ig isotype found in serum, similar to IgM in mammals. In the intestinal mucosa, both IgM and IgT have been detected. However, it has been suggested that IgT have a specialized role in mucosal immunology similar to IgA in mammals [18]. T cells are detected in the teleost intestine of teleost fish species, including sea bass [23], carp [29], and zebrafish [30], and are abundantly located in the lamina propria and epithelium. Macrophages have been morphologically described in a number of teleost fish species [12, 31], and macrophage-like cells are often seen in between intestinal epithelial cells and in the lamina propria [32].

1.2.2 Antigen uptake in the teleost intestine

In their embryological development, teleost fish start out with an intestine formed as a straight tube without a defined stomach. During larva development, 85% of teleost fish species develop a stomach, while 15% lack a stomach or a region with low pH and predigesting [6]. When fully grown, the intestine is traditionally divided into three segments, the anterior, second, and posterior segments [1, 33]. The stomach and uptake of food is usually found in the anterior segment [34]. Previous studies have further identified the second segment of the gut as the main area for antigen uptake in the teleost intestine [6, 13, 35, 36]. Anal intubation with ferritin, *Vibrio anguillarum*, and horseradish peroxidase (HRP) in carp [13, 35] and rainbow trout [35, 36] showed uptake across the epithelium in the second segments and detected antigens in macrophages.

In mammals, antigens are transcytosed across the epithelium by M cells, located above PPs in the small intestine [7]. M cells are so far not shown in the teleost intestine. However, recent studies in the salmonid intestine identified specific M cell-like cells in the second segment of the intestine [37]. These antigen sampling cells showed a more rapid and higher uptake of particular antigen (10 nm BSA-gold) compared to other cells in the epithelium of the anterior intestine. Furthermore, uptake was also identified in a few dendritic-like cells in the posterior segment. Studies of the second segment under scanning electron microscopy (SEM) revealed cells in the same area with an M cells like surface [7]. As there are no specific antibodies for targeting mammalian M cells, their characteristic lectin binding properties has been used for identification [38]. Lectins are a structurally diverse group of proteins which bind reversibly to specific carbohydrate residues [39]. The lectin *Ulex europaeus* agglutinin (UEA-I) binds to α -L-fucose, and has shown to almost exclusively bind to the apical surface of mouse M cells [40]. Fuglem *et al.* found cells in the salmonid intestine positive for UEA-I binding localized in the same region as the BSA-gold- positive cells [37]. These cells were also negative for the lectin wheat germ agglutinin (WGA), which binds N-acetylglucosamine and sialic acid, and typically binds to mammalian goblet cells.

1.3 Vaccination in Aquaculture

Aquaculture is defined as the farming of plants and animals in all aquatic environments, and it is one of the fastest growing food producing sectors worldwide. Aquaculture now accounts for almost half of all the world's food fish, and as a rich source of proteins, essential fatty acids, and micronutrients, farmed fish is an important part of people's everyday diet (FAO, 2014). With a growth in fish farming industry and farmed fish accounting for such a large part of the world's food consumption, it has become a pressing issue to increase efficacy of production and to minimize any kind of losses. Atlantic salmon has become one of the most important farmed fish species within aquaculture. In 2012, over 1.2 million tons of farmed salmon was produced in Norway, which corresponds to an increase 300% in production from 2000 (0.4 mill ton) (SSB, aquaculture 2014). However, in the period 2011-2012, over 5% of all farmed salmon in Norway was lost to different diseases after they were transferred in sea pens (Mattilsynet, 2014). Disease prevention is especially important when farming animals at high densities, and vaccination has so far proven to be the most effective and environmental friendly method of combating disease in aquaculture.

1.3.1 Vaccines

Vaccines are any preparation used to establish or improve immunity against a particular disease or group of diseases. Vaccination is achieved by administration of antigenic material that will activate the adaptive immune system and induce an antigen-specific adaptive immune response, including production of antibodies. There are several strategies to produce vaccines, and preparations can be made from attenuated or inactivated pathogen, protein/peptide conjugates (subunit vaccines), bacterial toxins, DNA, or recombinant vectors expressing pathogen antigens. Already in 1942, a successful vaccination against *Aeromonas salmonicida* was reported in cutthroat trout by oral administration of inactivated bacteria [41]. Vaccination decreased mortality by 50% compared to untreated fish when challenged with the pathogen. However, little attention was given to fish vaccine in the next decades, as large-scale use of antibiotics were introduced into agriculture in the 1940's. Soon after, resistance to antibiotics was observed in bacteria, bringing attention to alternative treatments such as vaccination [42]. This resulted in the first fish vaccine licensed in the USA in 1976. It consisted of orally administrated killed *Yersina ruckeri*, which provided efficient protection against enteric redmouth disease in farmed rainbow trout [43]. In the period 1987 to 2002,

Norwegian salmon farming lowered the amount of antibiotics and chemotherapeutics from 48 tons to 1 ton. At the same time production of farmed salmon increased from approximated 56 000 tons to 533 000 tons [44]. This immense reduction of antibiotic use in Norwegian salmon farming, and aquaculture in general, is due to the successful introduction of disease prevention by vaccines.

Today there are a number of commercially available fish vaccines, especially for salmon. For bacterial diseases like furunculosis (*Vibrio salmonisida*), vibriosis (*Vibrio anguillarum*), and coldwater vibriosis (*Aeromonas salmonisida*) commercial vaccines are available and widely used (Pharmaq). In addition, vaccines against some of the more common viral diseases, like infectious salmon anemia (ISA), infectious pancreatic necrosis (IPN), and pancreatic disease (PD) are also available (Pharmaq, MSD animal health). Conventional vaccine designs are most commonly based on inactivated whole bacterial cell or virus preparation together with an oil adjuvant [45]. The most successful vaccines used today (including those mentioned above) are based on this approach.

1.3.2 Administration of vaccines

At present, fish vaccines are exclusively administrated through injection or immersion. Injection vaccines are either injected into the peritoneal cavity (intraperitoneal) or into the muscle (intramuscular), depending on the type of vaccine. The advantages with injecting vaccines are that a small, known amount of antigen can be delivered systemically to induce a strong systemic immune response. Often, injection vaccines are found to be more effective and give a prolonged protection compared to other ways of administration, as several vaccines/antigens formulated with an adjuvant can be injected simultaneously (multivalent vaccines). However, there are many limitations and side effects to this administration route. Vaccination requires handling, anesthetizing and injection of fish, causing stress for the fish. In addition, injections are labour intensive and costly for the farmer. In salmon farming, vaccines have to be administered before fish are transferred to sea pens, as the effort and costs of injecting individual fish in the pen are too large.

Fish under a certain size (farmed salmon under 20g) are difficult to inject and the loss from handling is greater. However, young fish are more often susceptible to disease [43].

Immunization by bath immersion is one of the easiest ways vaccination that requires no handling and is therefore often a used method of administration in small fish. Commercial

vaccines against bacterial pathogens like *Vibrio spp.* are available (Pharmaq), but immune protection against a majority of fish pathogens provided by immersion vaccination is generally less robust compared to injection vaccines [16].

Oral vaccination is often considered as the most desirable way of administering vaccines in fish farming. Delivery of vaccine formulations in or together with fish feed offers the advantage of being stress free and easy to administer to a large number of fish at the same time. This way, vaccines can be administered without considering the size and location of the fish and protection can be easily improved by vaccination boosts even after the fish are transferred into sea pens. Furthermore, oral administration targets directly the intestinal mucosa and can induce local adaptive immune responses as well as systemic immune responses [46]. This is beneficial, as mucosal surfaces are the main port of entry for most pathogens. However, even though the first fish vaccines were administered orally [43], oral vaccination is still associated with a need for large amount of vaccine formulation and lower protection compared to injection vaccines. In addition, protection against degradation of vaccine antigens is a major concern, as the intact antigen needs to pass through the intestine to reach the second segment [6], where they have to cross the epithelium and activate the mucosal immune system. There is still relatively many gaps in the knowledge of teleost mucosal immunology, especially on how antigen are sampled and processed in the teleost intestine. Therefore, more research is necessary especially to be able to develop effective oral vaccines in the future.

1.3.3 Adjuvants

Adjuvant are defines as a group of structurally heterogeneous compounds able to enhance the immunogenicity of a vaccine [47]. They are often a necessary component in vaccine preparations to achieve a prolonged and enhanced protection against diseases. In fish, vaccines formulations contain mostly different types of mineral oil as adjuvants. Oil based adjuvants show several unwanted side effects like injection site lesions and internal adhesion between organs or organs and the peritoneal wall. Most severely, oil based adjuvants were further shown to induce autoantibodies and autoimmune responses [48].

Adjuvants can be classified by their chemical or physical nature. However, as related compounds can have different immunogenic properties, classification can also be based on the mechanisms induced by the different compounds. Adjuvants are then categorized in a two-

signal model, in which the presentation of antigen (signal 1) and additional secondary signals (signal 2) are both important for activation of the adaptive immune system [45]. Signal 1 facilitators enhance and optimize presentation of vaccine antigens to the adaptive immune system by increasing and prolonging the availability of antigens. Examples of signal 1 facilitators are delivery systems enabling slow and sustained release of antigen such as oil emulsions, including Freund's complete and incomplete adjuvants, and nano/microparticles. Nano/microparticles encapsulating vaccine antigens is a field gaining more and more interest as an alternative to oil emulsion.

Signal 2 facilitators are immune-stimulants that directly act on the immune system to induce and increase the immune response to the antigen presented in the vaccine [49]. Examples of signal 2 facilitators are aluminium salt-containing adjuvants (Alum) that induce mostly humoral (Th2-helper cell-biased) responses and are one of few adjuvants approved for use in human vaccines [50]. Lipoproteins and lipopeptides are found in a number of microorganisms and are strong signal 2 facilitators that induce strong and long lasting inflammatory responses in mammals. Some adjuvants can also be categorized as signal 0 facilitators (or as a sub-category of signal 2 facilitators). These compounds mimic pathogen-associated microbial patterns (PAMPs) that trigger pathogen recognition receptors (PRRs) expressed by innate immune cells, such as Toll-like receptors (TLRs), NOD-like receptors, dectin-1 or RIG like helicases [45].

1.4 Nano and microparticles as adjuvants

Nano- and microparticles have become a promising alternative to oil adjuvants as carriers for vaccine delivery. Furthermore, the large number of materials and methods, which can be used to produce such particles and versatility in characteristics of these particles, make them especially interesting for future development of oral vaccines. Antigens and immunostimulatory agents can be incorporated into particles by covalent linking or physical entrapment. The antigen is released into the environment as the particle disintegrates [51]. Encapsulation of vaccines in nano/micro-particles helps to overcome many of the obstacles seen with oral vaccines, such as incorporation and storage of the vaccine in the fish feed, protection of antigens against degradation in the intestine, and give a prolonged release of antigen. In addition, immune response can be enhanced by using a particle matrix that can act as a signal 2 facilitator. Several compounds have been studied for the development of oral

vaccines, both in mammals and fish. Compounds like alginate, poly(lactic-co-glycolic acid) (PLGA), chitosan or liposomes have been successfully tested, but so far no nano-/microparticle based vaccine has been licensed for vaccination in fish [18].

Chitosan is a nontoxic, biocompatible, biodegradable, natural polysaccharide converted from chitin by deacetylation. Chitosan is good candidate for vaccine encapsulation, as it can easily be chemical modified or conjugated with other compounds and is strongly muco-adhesive, which results in increased binding to mucosal epithelia [52, 53]. The adjuvant effect of chitosan was first demonstrated in the 1980s, showing that chitin and chitosan can act as a signal 2 adjuvant by activating the NLRP3 inflammasome [54, 55]. Chitosan has been used as a delivery carrier for DNA-vaccines by oral administration in fish, proving that the DNA-chitosan complexes are taken up and can cross over the intestinal epithelium [56].

Polyinosinic polycytidylic acid (Poly I:C), a double stranded polyribonucleotide that mimics a viral infection, is another signal 2 facilitator. It is recognized by TLR3 and cytosolic RIG like helicases and induces a pro-inflammatory immune response including type 1 interferons (IFN) production in many species, including fish [57]. IFNs are cytokines secreted by immune cells in the early phase of the defence against viral infections, and Poly (I:C) is therefore inducing a non-specific antiviral state in the host. The protective capability of Poly (I:C) was proven in rainbow trout infected with infectious hematopoietic necrosis virus (IHNV). Fish injected with Poly (I:C) were protected against IHNV when challenge 2 days after injection, and IHNV-specific antibodies were detected in the survivors [58].

Poly lactide co-glycolic acid (PLGA) particles have been widely explored for potential use in oral vaccines. PLGA is a hydrophobic, biodegradable and non-toxic polyester that is easy and cheap to produce [59, 60]. PLGA is a signal 1 adjuvant that facilitates slow and sustained release of encapsulated or conjugated antigen. PLGA particles, containing outer membrane proteins from *Aeromonas salmonicida*, were injected into *Labeo rohita* (roho). The results were compared to injections of bacteria whole cell preparation, Freud's incompetent adjuvant with outer membrane proteins, and the outer membrane proteins alone. After 21 and 41 days, PLGA injected groups showed significantly higher stimulation of both specific and non-specific immunity. Indicating the beneficial use of the polymer as a drug carrier [61]. Furthermore, Human gamma globulin (HGG) encapsulated in PLGA particles (PLGA/HGG) was orally intubated in Atlantic salmon, and PLGA/HGG was detected in serum up to 5 weeks after intubation [62].

1.5 The zebrafish as a model

1.5.1 The Zebrafish model

Zebrafish (*Danio reiro*) is a small freshwater fish naturally found in India, Pakistan and Bhutan. It is a well-characterized model organism in biology, and is widely used in labs all over the world. Zebrafish are teleost fish that are easy to keep, have a short generation time, and produce a large number of offspring. One of the benefits with zebrafish, are the optically transparent embryos and larva, which make it a good model for developmental biology and study of human diseases [63, 64]. Zebrafish have been proposed as a model for Crohn's disease and inflammatory bowel disease [65, 66], and used to study infectious diseases like herpes simplex virus and tuberculosis [67, 68]. In addition, mutagenesis can easily be performed and today many transgenic zebrafish lines are available (Zebrafish International Resource Center (ZIRC)), adding a powerful tool to the zebrafish model.

As adults, zebrafish have both an innate and adaptive immune system. The innate immune system, with well-characterized macrophages and neutrophils, are functionally active in embryos and early larva. The zebrafish complement system is also highly developed, as is common in fish species [69]. The adaptive immune system is characterized by B- and T- cells, and is found to be functional first after 4-6 weeks post fertilization [70]. Immune homologs, such as immunoglobulin light chain, T cell receptor, MHC class I and II, and Rag 1 and 2 are confirmed in zebrafish [71-73].

Zebrafish is therefore also a good model for uptake studies in the teleost intestine and studies indicate that antigen sampling cells are located in the second segment of the zebrafish intestine, as seen in other teleost species. These cells can be identified by supranuclear vacuoles, where macromolecules taken up from the intestinal lumen were shown to be located [74, 75]. However, it is not clear if these antigen sampling cells have the same ability, as M-cells in mammals, to sample antigens and whole microbes and passing them to APCs. In addition, zebrafish can be raised under germ-free conditions, making it an interesting model for studying interactions between host and both commensal bacteria and pathogens in the intestine [74].

1.5.2 **Mycobacterium marinum** infection

M. m is a rod shaped bacterium with a waxy and hydrophobic surface that is a natural pathogen in fish and other ectotherms. The bacteria cause a systemic granulomatous disease in its host [76, 77]. The aerobic bacteria infects aquatic animals and therefore has an optimal growth temperature between 25 – 35 °C with a generation time of 6-8 hours [78]. *M. m* is not a natural pathogen for humans, as it does not grow well at 37 °C. Nevertheless, infectious lesions can be found in the cooler, superficial regions of the human body, such as the extremities, often referred to as swimming pool granulomas. In very rare cases, *M.m.* can cause systemic infection [79].

Mycobacterium tuberculosis (*M.tb*), a close relative to *M.m*, is the causative agent of tuberculosis (TB) in humans. Many cell-biological processes and mechanisms involved early in *M.m* infection in zebrafish are similar to human *M.tb* infection in humans, making the zebrafish a valuable and powerful model for *M.tb* infection and TB treatment. One of the hallmarks of human TB is the formation of organized granulomas with a necrotic core containing *M.tb* bacteria [80], which is also seen in *M.m* infected zebrafish, in contrast to mouse TB-models. Both bacteria are phagocytosed by the hosts macrophages, inhibiting maturation of phagosomes into phagolysosome [77].

The group of Lalita Ramakrishna were pioneers in establishing *M.m* infection in zebrafish larva as a model system for studying cellular and molecular processes involved in early TB [80]. Bacteria can be visualized in the transparent embryos by using fluorescent strains of *M. m*, and co-localization with macrophages (granulomas) and neutrophils can be seen in transgenic zebrafish lines expressing fluorescent proteins cell-type specifically [81, 82]. However, the natural infection route of *M.m* in adult zebrafish is not clarified, although there are strong indications that the primary route of infection is via the intestine [83].

2 Aims of the study

This master's thesis aim was to (1) establish a zebrafish model system for testing nano/microparticles as potential vaccine carriers for teleost fish oral vaccination, and (2) investigate pathogen infection through the teleost intestine. The zebrafish model was established by using fluorescent particles, samples sectioning, histochemistry, immune-histochemistry, and light and confocal laser microscopy. Pathogen infection was investigated using fluorescent bacteria.

3 Materials and methods

3.1 Zebrafish care and husbandry

Adult zebrafish used in the experiments in this thesis came from our facility at the University of Oslo, or were obtained from the zebrafish facilities at the Norwegian University of Life Sciences (NMBU). The aquaria were kept at a constant 28°C, with continuous circulation of tank water containing Instant Ocean® sea salt (United Pet Group, Blacksburg, VA, USA), calcium chloride, and sodium bicarbonate. Approximately 10% of the total system water volume was exchanged daily, and the light: circle was kept at 12:12 hours. Fish were fed three times daily, twice with brine shrimp and once with SDS 400 dry feed.

All experiments were done on adult, wild type zebrafish, age between one and a half and two and a half years. Fish were transported from the aquaria to the lab in a plastic tank and stored at 28°C in a dark incubator (Thermo Scientific Heratherm General Incubator) for 1h before experiments to allow the fish to calm down, and ensuring they were as stress free as possible before experiments. Ethical permission was obtained from the Norwegian national animal research authority (FOTS permission ID: 7276).

3.2 Oral intubation of adult zebrafish

The protocol for intubating adult zebrafish was modified as outlined in [84]. Prior to the procedure fish are starved for 24h to ensure an empty intestine. One fish at a time was sedated in a separate container with 150mg/L Tricane (Sigma-Aldrich, St. Louis, MO, USA) in system water until they were no longer were able to stay upright and did not respond to tail pinching [85]. The sedation time was kept to a minimum (1-2 minutes), and did not exceed 3 minutes, to prevent eventual damage caused by the anaesthetic. Properly sedated fish were then removed from the sedation container and placed in a groove of a sponge soaked in system water with the head protruding slightly from the sponge.

For the intubation, a 50µl Hamilton Microliter™ syringe (Hamilton Company, Nevada, USA) with a 2 cm long rubber tube (Perifix® Soft 500 Filter Set. B. Braun, Melsungen, Germany) attached to the tip was used. The syringe was filled with a PBS solution containing particles or bacteria and approximately 1 cm of the rubber tube was inserted into the sedated fish's

mouth, carefully pushing the jaws apart. Ensuring that the tube is passed the gills and reached the anterior part of the intestine, 5µl of the solution was injected and the tube slowly removed.

After oral intubation, fish were immediately removed from the sponge and placed in a new container with fresh system water for recovery. Then, a new fish was sedated and the intubation repeated. All fish that showed any signs of injuries (e.g. bleeding, slow swimming, inability to keep upright) during recovery, were euthanized within 5 min after intubation as described below.

Fish were kept at 28° C in an incubator and euthanized after defined time points (5min, 1h, 3h and 5h) with an overdose of tricaine (300 mg/ml). Fish were left in the tricaine solution for at least 10 min to ensure that fish were properly dead, as observed by termination of opercular movement, and inability to stay upright. For each time point a least three fish were sacrificed in all experiments. No fish were kept alive for more than 5h after intubation.

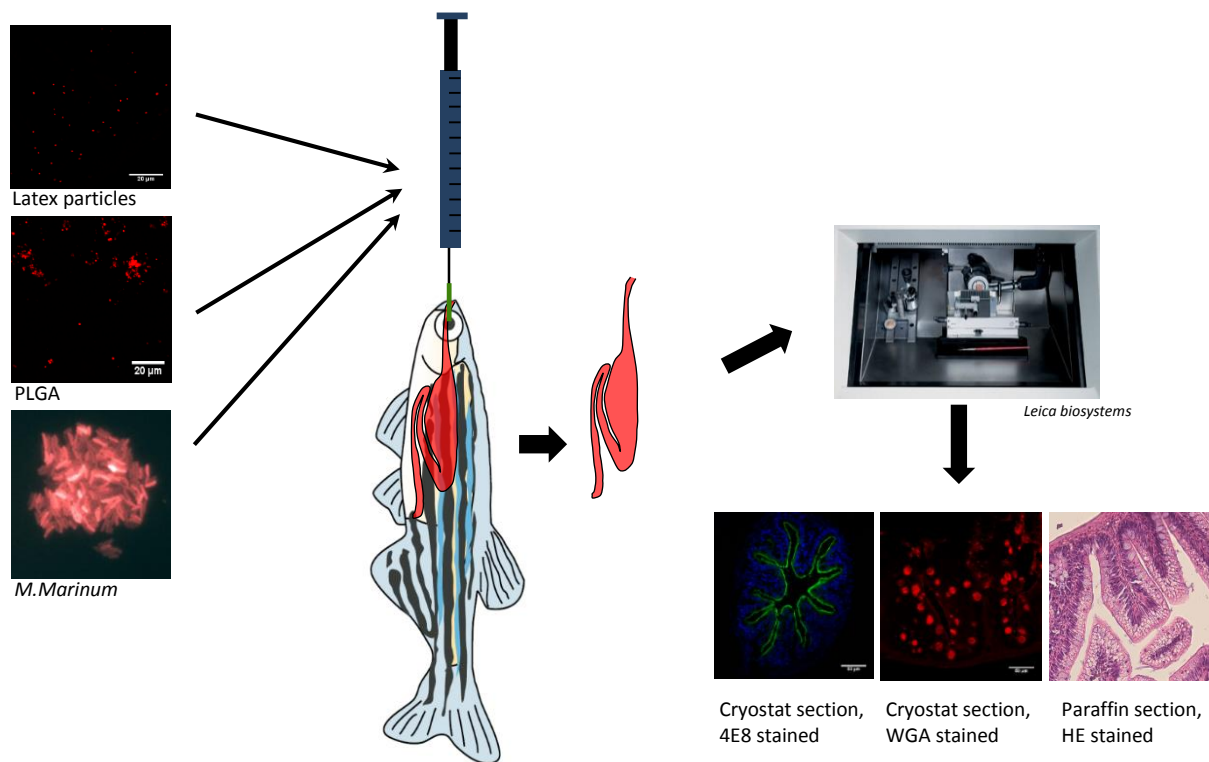


Figure 2: Oral intubation of adult zebrafish. Fluorescent particles or bacteria were directly injected into the intestine of sedated zebrafish by oral intubation. After set time points, organ samples were dissected out and prepared for paraffin or cryostat sectioning. As illustrated by cryostat sections of the intestine stained with antibody 4E8 and WGA-lectin, separately, and a paraffin sections of the intestine stained with HE.

3.3 *Mycobacterium marinum*

3.3.1 Culturing *Mycobacterium marinum*

M.m was cultured and prepared for oral intubation of zebrafish as outlined in [67, 78]. A *M. m* strain with a plasmid coding for the red fluorescent protein dsRed and kanamycin resistance (msp 12::deRed2, gift from Lalita Ramakrishnan, University of Washington, Seattle, WA, USA) was used. Liquid culture media was prepared from Difco Middlebrook 7H9 broth and supplemented with 0,02% glycerol, 10% ADC (Albumin, dextrose, catalase) and 0,05% tween 80. Kanamycin (Sigma-Aldrich, St. Louis, MO, USA) was added at 50 µg/ml to select for bacteria bearing the plasmid. Liquid *M.m* culture was prepared by adding 5µl of a frozen stock into media and incubated at 32 °C on a shaking stage in the dark.

3.3.2 Preparation of *Mycobacterium marinum* for oral infection

For oral infection, *M.m* was harvested in the exponential growth phase with an OD₆₀₀ between 0.5 and 0.9. The bacterial culture was re-suspended twice using a 22-gauge needle and a 20ml syringe to disrupt any bacterial aggregates prior to OD₆₀₀ measurement. The 10 ml bacteria culture were centrifuged at 3000g for 10min. The medium was removed and bacteria were washed twice with 5ml PBS. Finally, bacteria were re-suspended in 1 ml of PBS and passed through a 27-gauge needle 10 times before oral intubation.

3.4 Sample preparation and sectioning

Zebrafish were sacrificed at set time points after oral intubation and organs were quickly dissected out as outlined in [86]. The organ package (intestine, liver, spleen, pancreas and gallbladder) was taken out as a whole to avoid damage to the organs. Mice were sacrificed with cervical dislocations, the small intestine dissected out and 0.5 cm samples containing Peyer's patches were cut out. Samples were then prepared for different applications as described below. Alf Seljenes Dalum at NMBU provided samples of salmon intestine, mice were provided from the animal facility at IBV, UIO

3.4.1 Preparation for cryostat tissue sections

For cryostat sections, the samples were fixed in 4% paraformaldehyde for 2h at 4°C with an approximately volume ratio of 1:20 (tissue: fixative). After fixing, the tissue was washed with PBS 3 times for 5 min to remove any fixative, organs were separated, and connective tissue removed. The intestine was kept either complete or divided into three segments (fig 3; anterior, second and posterior segments). To freeze samples, small molds were prepared with aluminium foil and filled with Tissue-Tek® O.C.T. Compound (Sakura® Finetek, Tokyo, Japan). Samples were placed in the mold, completely embedded in Tissue-Tek® O.C.T, and marked with the type and orientation of the sample. The samples were then shock frozen in liquid nitrogen and stored at - 80 °C. To ensure optimal sections, different approaches for sample preparation were tested (results not shown). Fixed samples gave less background fluorescence compared to unfixed samples, and sucrose infiltration was found to be an unnecessary step as tissue preservation was not improved.

All cryostat sections were made on Cryostat Leica CM3050 S at the NMBU, histology lab. The sections were cut at a thickness of 7µm and were picked up on Superfrost™ plus slides. 3-10 serial sections were picked up on one slide and air dried for 30 min before they were either stained or stored at -20°C until later use.

3.4.2 Preparation for paraffin sections

For paraffin sectioning, the samples were fixed in 10% neutral buffered formalin overnight (o/n). For paraffin infiltration a Shandon excelsior™ tissue processor with a standard paraffin infiltration program was used overnight, where samples were dehydrated through a series of graded ethanol baths, followed by sample clearing through xylene baths. The paraffin wax was infiltrated at 58°C over two sessions. After infiltration, samples were cleaned and separated into different organs before embedded in a paraffin block. The samples were sectioned on a Thermo Scientific Sliding Microtome Ticrom HM 450 to a thickness of approximately 5µm, and picked up on ThermoScientific™ Polysine Adhesion slides. Sections were stored at RT in the dark.



Figure 3: Illustration of the different sections of the zebrafish intestine. Anterior segment correspond to section 1 and 2, the middle/second to 3, 4, and 5, and the posterior to 6. Figure taken from Oehlers et al. [1]

3.4.3 Preparation of resin-embedded tissue sections for Transmission Electron Microscopy (TEM)

To visualize uptake of 10 nm BSA-gold particles in the zebrafish intestine we used TEM. The protocol used for TEM sample preparation is standard for the Electron Microscopy lab, UIO.

Samples of the zebrafish intestine were fixed in 2.5% glutaraldehyde and 4% formaldehyde in 0,1M cacodylate buffer o/n. Thereafter they were post-fixed with 1% osmium tetroxide and rinsed with distilled water. For resin embedding, samples stained for contrast with 1% uranyl acetate, dehydrate with different concentrations of acetone, infiltrated with different concentrations of resin in a microwave (PELCO BioWave®), as outlined in table 1. After infiltration, samples are transferred into molds with fresh resin and polymerized o/n at 60°C.

Step	Reagent	Time	Watts	Temp (°C)
1	Water	60 sec	250	37
2	Uranyl Acetate 1%	3 min	250	37
3	Uranyl Acetate 1%	3 min	--	RT
4	Uranyl Acetate 1%	3 min	250	37
5	Water	60 sec	250	37
6	Water	60 sec	250	37
7	Water	60 sec	250	37
8	50% Acetone	60 sec	250	37
9	70% Acetone	60 sec	250	37
10	90% Acetone	60 sec	250	37
11	100% Acetone	60 sec	250	37
12	100% Acetone	60 sec	250	37
13	100% Acetone	60 sec	250	37
14	Resin 25%	3 min	350	45
15	Resin 50%	3 min	350	45
16	Resin 75%	3 min	350	45
17	Resin 100%	3 min	350	50
18	Resin 100%	3 min	350	50
19	Resin 100%	3 min	350	50

Table 1: Protocol for contrasting and resin-embedding of tissue in a microwave.

3.5 Histochemistry and immuno-histochemistry

3.5.1 Preparation of cryostat sections

Before any kind of staining, slides with cryostat sections were thawed in RT for 15-30min and rehydrated in a glass slide-holder containing PBS for 10 min at RT.

3.5.2 Hematoxylin and Eosin stain (HE)

Hematoxylin and Eosin (HE) stain were used for recognizing various tissue types and the morphologic differences in tissue sections. Hematoxylin has a deep, blue-purple color that stains nuclear acids. Eosin has a pink color that stains proteins in a nonspecific manner [87].

Paraffin sections were deparaffinise using Xylene and stained with HE on a Tribune stainer HCS 33, automatic multiple slide stainer.

Cryostat sections were stained manually. Rehydrated slides were dipped in a jar containing Mayer's hematoxylin for 30sec, rinsed in water for approximately 1 min, and then dipped in 1% Eosin Y solution for 30sec. Sections were then dehydrated with two changes in 95%

alcohol and two changes in 100% alcohol. Alcohol was extracted with two washes in Xylene. Slides were dried and mounted with PVA.

3.5.3 Lectin histochemistry

Lectins are a structurally diverse group of proteins that bind specific carbohydrate-domains.

UEA-I binds α -L-fucose, and WGA binds N-acetylglucosamine carbohydrate groups.

Cryostat sections of the zebrafish intestine were stained with FITC-conjugated UEA-1 lectin (L9006 Sigma-Aldrich, MO, USA) and rhodamine-conjugated WGA (RL-1022 Vector laboratories, CA, USA) as described in [37].

Slides with cryostat sections were incubated in a blocking solution with 2% BSA in PBS Tween-20 (PBST) for 1h in room temperature. Thereafter, sections were stained with 10 μ g/ml UEA-1 or 2 μ g/ml WGA in blocking solution for 1h at RT. Slides were then washed 3x5min in PBS and counterstained with nuclear stain (Hoechst, 1 μ g/ml) for 10min. After 2x5min washes in PBS, slides were mounted with PVA under a cover glass.

3.5.4 Immuno-histochemistry with the Antibody 4E8

The 4E8 antibody recognizes an antigen at the apices of the microvilli (just above the actin rich area) and stains the brush border in the zebrafish intestine. The antibody does not stain the apices of goblet cells, and they are seen as gaps in the stained brush border. Cryostat sections of the zebrafish intestine stained with the antibody 4E8 as outlined in [88].

Cryostat sections were incubated with 10 μ g/ml primary antibody 4E8 in a blocking solution containing 10% goat serum, 2% BSA, 0,1% Triton-X PBS for 2h at RT or o/n at 4°C. Slides were washed 3x5min in PBS and incubated with a red fluorescent secondary antibody (cy2-conjugated goat anti-mouse, Jackson ImmunoResearch, PA, USA), in blocking solution for 2h at RT. After, sections were washed, stained with nuclear counter stain and mounted as described for lectin stained sections.

3.5.5 Immuno-histochemistry with Anti-L-plastin

To identify leukocytes in the intestine, liver and spleen, an antibody against L-plastin was used. L-plastin is a pan-leukocyte specific protein that cross-links actin filaments into tight bundles, increasing the stability of actin-based structures. L-plastin has been identified as an important component in cellular processes critical for neutrophils, macrophages, osteoclast, eosinophil, and T- and B-lymphocytes [89]. The polyclonal rabbit antibody against L-plastin was kindly provided by the group of Annemarie H. Majer, Leiden University, Germany. The protocol for antibody staining is modified from [90].

Cryostat sections were blocked for 1h in room temperature with a blocking solution containing 2% BSA and 0,1% triton-X in PBS. Thereafter, the sections were incubated with primary antibody (1:500) in blocking solution overnight at 4°C. After 3x10min wash in PBS, sections were left in blocking solution for 1h and incubated with a secondary antibody (1:200, cy2-conjugated goat anti-rabbit, Jackson ImmunoResearch, PA, USA) in blocking solution for 2h at RT. Sections were then washed, stained with nuclear counter stain and mounted as described for lectin stained sections.

If kept dark and at 4°C, the fluorescently stained sections could be investigated with the confocal microscope up to at least two weeks after staining without loss of fluorescence intensity.

3.5.6 Correlative confocal laser and light imaging

Fluorescently labeled sections give a limited description of the morphology in sections, as fluorescent markers and the amount of channels on the microscope restrict it. To obtain a better overview of the sections, a correlative confocal laser and light microscopy imaging method was developed.

Cryostat sections were prepared and labeled for confocal laser imaging as described over. The orientation and placement of imaged sections were noted in relationship to the glass slide. Also, a water-soluble adhesive, like PVA, was used to ensure that the cover glass could be removed without damaging the sections. After imaging, the glass slides were soaked in PBS for 2h in RT, or until the cover glass slip off when slides are removed from PBS. The re-hydrated sections were then HE stained as described above. HE staining of the sections destroyed the fluorescence from particles and antibodies.

3.6 Imaging

Imaging of HE stained sections, both cryostat and paraffin, were acquired with a Microscope Axio Imager.M2 (Zeiss, Oberkochen, Germany) at NMBU. Fluorescence images of stained cryostat sections were acquired with an Olympus FluoView 1000 inverted confocal microscope at The Oslo NorMIC Imaging facility at IBV, UIO. Image of resin sections were acquired with a Phillips transmission EM (CM200; Phillips, Eindhoven, The Netherlands). All images were processed with ImageJ (Image Processing and Analysis in Java).

3.7 Nanoparticles used for oral intubation

3.7.1 Carboxylated latex particles (CLPs)

To establish the zebrafish model, 0.5µm latex beads, carboxylate-modified polystyrene, with red fluorescent (L3280-1ML, Sigma-Aldrich, St. Louis, MO, USA) and 1µm FluoSpheres® carboxylate-modified microspheres (Invitrogen) were used. The particles have a strong red fluorescent signal with an excitation maximum at 580nm, and are easily detected on cryostat section under the confocal microscope.

To calculate the approximate number of particles suspension, a formula from Fluoresbrite® Microparticles (Polyscience, Inc. technical data sheet 431) was used.

$$\text{Number of particles per ml} = \frac{6W \times 10^{12}}{\rho \times \pi \times \varphi^3}$$

w = grams of polymer per ml in latex (0.02g for 1µm particles and 0.025g for 0.5µm particles used in experiments)

φ = diameter in microns of latex particles

ρ = density of polymer in grams per ml (1.05 for polystyrene)

Calculations for number of particles per ml in stock solution:

$$1\mu\text{m} = 3.6378 \times 10^{10} \text{ p/ml}$$

$$0.5\mu\text{m} = 3.6378 \times 10^{11} \text{ p/ml}$$

3.7.2 Preparation of Chitosan poly (I:C) nanoparticles

Chitosan is a biodegradable, biocompatible, mucoadhesive, and non-toxic polysaccharide with adjuvant properties [52]. It has been widely applied in pharmacy and medicine, and several studies have tested chitosan as a drug carrier for oral administration in fish [56, 91]. Polyinosinic polycytidylic acid (Poly (I:C)) is a double stranded polyribonucleotide that mimic viral infection and induce a type I IFN in many species, including fish. The protocol for preparation of the chitosan-poly I:C (Chi/pIC) nanoparticles (NPs) is already established in our group, and the particles have been tested in cell culture and adult zebrafish [92].

To prepare Chi/pIC NPs, low molecular chitosan (Kitozyme, Herstal, Belgium), conjugated with the near-infrared fluochrom IRDye® 680RD (LI-COR Biotechnology, NE, USA) was dissolved in 1% acetic acid/dH₂O at a concentration of 5 mg/ml overnight. The stock solution was diluted to 2 mg/ml, pH adjusted to 6 with 5 M NaOH and sterile filtered. A cryostat stock of poly (I:C) were thawed, diluted to 1 mg/ml with 0.9% NaCl and reannealed at 72 °C for 10 min. 500 µl of 1 mg/ml poly (I:C) solution were added dropwise to 500 µl of 2 mg/ml Chitosan under stirring, and the solution was left stirring for another 20 min. The nanoparticles were collected in a 1.5 ml Eppendorf tube on a 20 µl glycerol bed and centrifuged at 10000g for 10 min. The supernatant was discarded carefully and NPs were re-suspended in 0.45% NaCl and finally dissociated by water bath sonication for 10 min before use.

3.7.3 PLGA nanoparticles

PLGA nanoparticles were prepared and kindly provided by Federico Fenaroli according to a protocol established in our group [93].

In brief, particles were prepared by standard oil in water emulsion technique. PLGA (50:50 RESOMER® RG 500, Evonik Röhm GmbH) and rhodamine (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dichloromethane (DCM) and stirred o/n. For the water phase, polyvinyl alcohol (PVA) was dissolved in water at a concentration of 0.001 % (w/v). The two solutions were mixed and sonicated to obtain a primary emulsion, and kept stirring o/n to evaporate DCM. Particles were then washed twice with water and collected via ultracentrifugation. Particles have a size range between 50 nm to 400 nm.

4 Results

4.1 Morphology of the zebrafish intestine

4.1.1 Difference in the epithelium along the zebrafish intestine

To investigate the morphology of the zebrafish intestine and to visualize the epithelium along the different intestinal sections, paraffin sections were stained with HE and investigated under light microscopy. The zebrafish intestine is a long tube, without a defined stomach, as seen stretched out in fig.4A (anterior to posterior from right to left). The intestinal folds are transversal in the anterior and second segment, and longitudinal in the posterior segment. They become shorter in an anterior to posterior direction (fig.4:A). In each fold, you can see the mucosa, which include the epithelium with simple columnar epithelial cells and underneath the lamina propria (fig.4: magnification of D, marked E). Underneath you find the muscularis with circular and longitudinal smooth muscle and the serosa (fig.4:magnification of D, marked M and S). Morphological differences in the segments can also be seen in the distribution of enterocytes and goblet cells. Goblet cells are found in all intestinal segments, identified as round structures with a light purple color in the epithelium of HE stained sections. The highest density of goblet cells are found in the second and posterior segments (Fig. 4: arrowhead in B, C, E, and magnification of D). In the second segments of the intestine specialized enterocytes with supranuclear vacuoles (SV) are detected, they are seen as “empty”, round structures in the epithelial cell cytoplasm (fig. 4: D). Previous studies have shown antigen sampling that cells can be identified by a large number of supranuclear vacuoles on the apical side of the cell [32, 75]. The SVs are found in almost all epithelial cells in the posterior part of the second segment (fig.4:magnification of D, arrow), and to a lesser extent in the anterior part of the second segment (fig.4:magnification of C, arrow). In the anterior and posterior segments (fig. 4: B, F), SVs are not present.

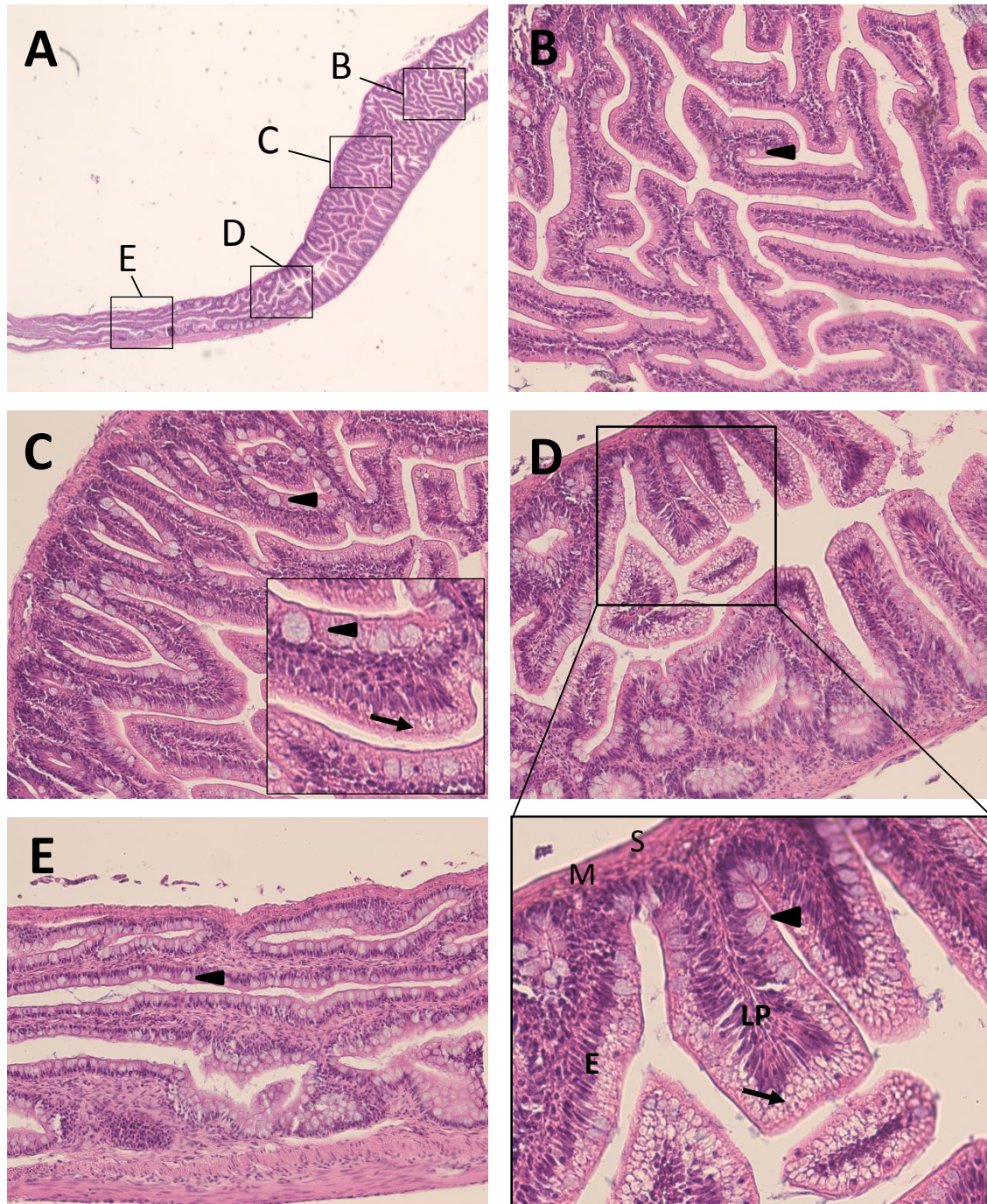


Figure 4: Paraffin section of the zebrafish intestine stained with HE. Magnified pictures of the intestine in A: Anterior (B), second (C and D), and posterior (E) segment. Goblet cells seen in all segments, indicated with arrowhead in B, C, E, and magnification of D. Supranuclear vacuoles are detected in the cytosol of epithelial cells in the second segment, as indicated by the arrow in C and magnification of D. The epithelium, lamina propria, muscularis and serosa are marked with E, LP, M and S, respectively, in magnification of D.

4.1.2 Fluorescent antibody staining

Studying the uptake of fluorescent particles in the zebrafish intestine, the epithelium and several mucosal immune components needed to be characterized by immuno-histochemistry. The 4E8 antibody stains an unknown antigen at the microvilli apices of absorptive cells bordering to the intestinal lumen of zebrafish [88]. This antibody was used to mark the border between the intestinal lumen and epithelial cells, marked with a green fluorescent conjugated secondary antibody (fig.5: A, B, C). Goblet cells are the only cell type not stained with 4E8, and can be seen as gaps in the green brush border along the epithelium (fig.5: B and C, red arrows). To stain leukocytes, an antibody against the pan-leukocyte marker L-plastin was used. Leukocytes are detected in high numbers along the LP (fig.5: D and E, arrowhead E) and as intraepithelial lymphocytes (IELs) (fig.5: E, arrows). In the spleen, leukocyte aggregations are found surrounding the central arterioles in the areas of the with pulp (fig.5: F and G, red circle), where blood is sifted through the spleen, and DC and Mφs are exposed to antigens [5]. In liver, leukocytes are detected spread around the tissue (fig4: H and I, red arrows).

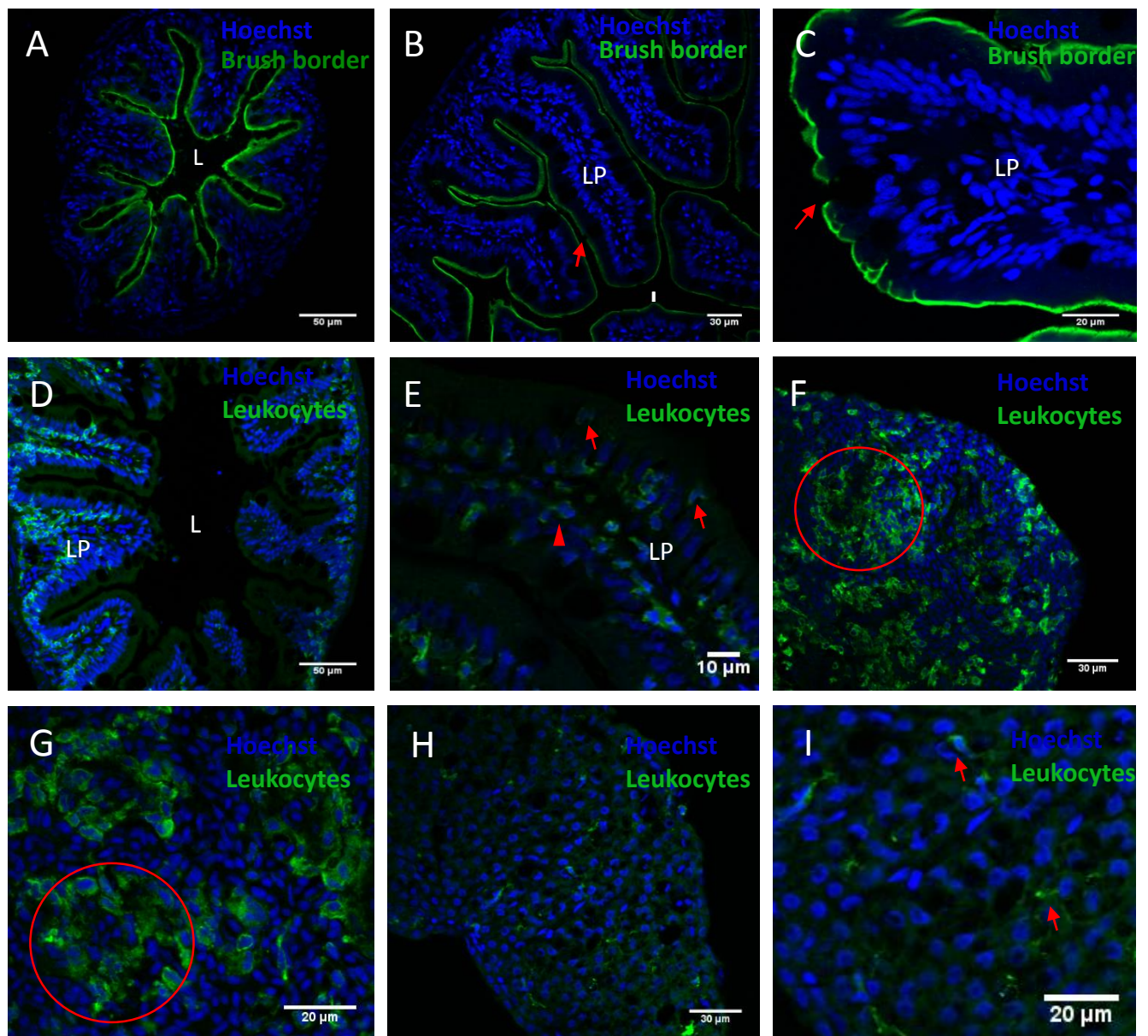


Figure 5: Zebrafish intestine stained with antibody 4E8 (A, B and C) and anti-L-plastin (D, E, and F), in addition to blue nuclear counterstain. Antibody 4E8 stains the apices of microvilli in the intestinal lumen), except goblet cells (red arrow B and C). Anti L-plastin detects leukocytes in the LP (arrowhead, E) and inside the epithelial layer (arrow E). F and G show anti-L-plastin staining of spleen with aggregation of leukocytes around central arterioles, the white pulp, marked with a red circles. H and I show leukocytes in liver, which are more spread and evenly distributed (red arrows). The intestinal lumen is indicated with L, and lamina propria with LP in the figure.

4.1.3 Identification of antigen sampling cells

In mammals, antigens and even whole bacteria can be transcytosed across the epithelial layer primarily via M cells, and presented to APC on the basal side [94]. M cells are part of the epithelia located over Peyer's patches. They can be identified by their short and irregular microvilli on the apical surface, high endocytic activity and often directly associated with APCs on the basal side [95].

An antigen sampling and presenting cells type equivalent to M cells are not detected in teleost fish, though antigen sampling cells are detected. A previous study by Fuglem *et al.* on the salmonid intestine, found that cells with antigen sampling characteristics in the second segment of the salmonid intestine were positive for UEA-I staining and negative for WGA staining [37], the same staining used to detect M cells in mammals [40]. To investigate if UEA-I staining on the apical membrane of antigen sampling cells is a conserved trait between salmonids and zebrafish, we stained sections of the zebrafish intestine with UEA-I and WGA. As a positive control, cryostat sections of mouse and Atlantic salmon intestine were stained simultaneously (n=3). All samples were positive for WGA staining, staining several cells along the mucosal surface (fig.6: A, B, C). UEA-I only stain mouse and Atlantic salmon samples (fig.6: E, F, H and I), and no staining could be detected in zebrafish intestinal epithelial cells (fig.6: D and G). UEA-I staining were tested along the whole zebrafish intestine, and all sections were negative as illustrated by a section from the second segment (fig.6:D and G), where potential antigen sampling cells was expected to be.

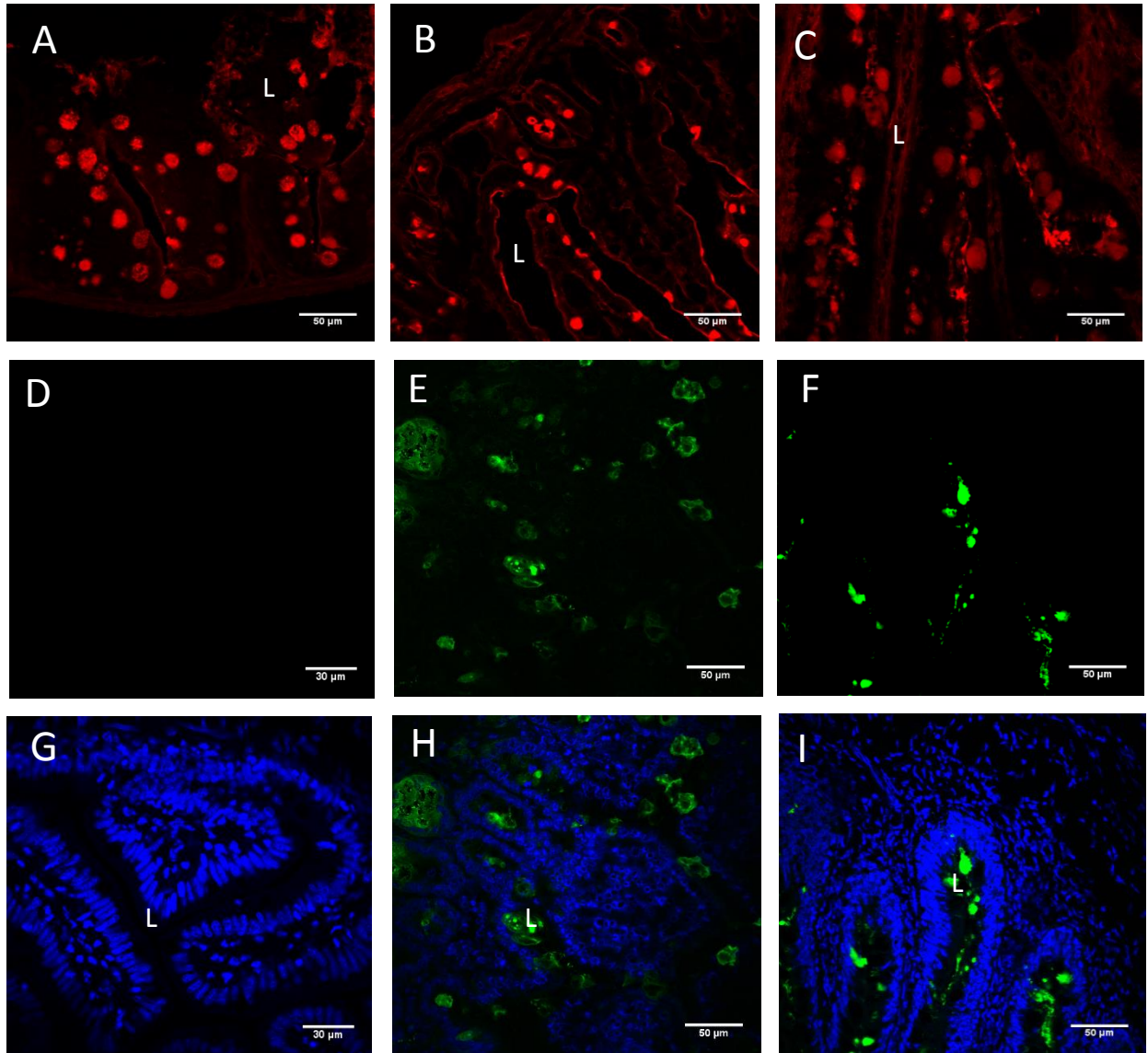


Figure 6: WGA (A-C) and UEA-I (D-I) lectin staining of zebrafish (A,D,G), mouse (B,E,H), and salmon (C, F,I) intestine. WGA (red) stains cells (goblet cells) in all samples. UEA-I positive cells (green) are only seen in the positive controls, whereas no staining was evident in the zebrafish gut. D-F show only the UEA-I-lectin channel, G-I show UEA-I stain and blue nuclear counterstain. The intestinal lumen is marked with L.

4.1.4 Oral intubation with 10nm BSA-gold particles

Another characteristic of antigen sampling cells in the salmonid intestine, seen by Fuglem *et al.*, is the rapid uptake of 10 nm BSA-gold particles by antigen sampling cells in the second segment [37]. In their study, samples of the salmonid intestine were dissected out and submerged in a solution containing 10 nm BSA-gold particles. Investigations under a microscopy with epipolarized light showed single cells or clusters of cells in the epithelium of the second segment containing large amounts of gold particles. They also found gold particles in cells with dendritic cell-like morphology underneath the epithelium, with arms protruding into the intestinal lumen.

We wanted to establish if antigen sampling cells in the posterior second segment of the zebrafish intestine show the similar characteristics. For that purpose, 10nm BSA-gold particles were injected into the zebrafish intestine using oral intubation and zebrafish were sacrificed after 1h. Samples of the second segment were embedded in resin, sectioned and investigated under a TEM. Gold-particles were detected in supranuclear vacuole-like structures in almost all epithelial cells, except cells at the bottom of the intestinal folds (fig.7: A-C). No particles were detected in cells underlying the epithelial cells (fig.7: E and F). There were no signs of irregular microvilli compared to cells without intracellular particles (fig.7: D)

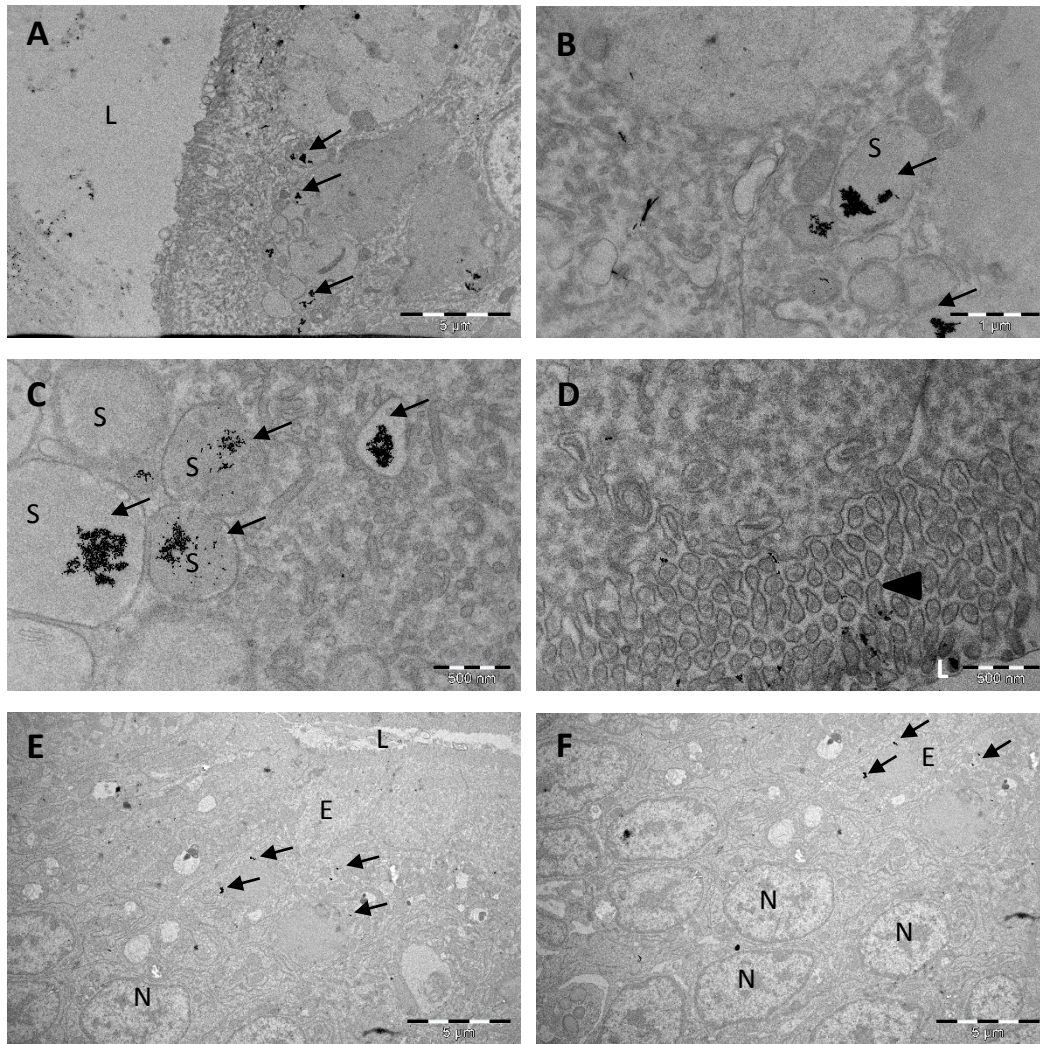


Figure 7: Resin sections of the zebrafish intestine 1h after oral intubation with 10 nm BSA-gold particles. The particles are seen as dense, black aggregates (arrow, A-C, E and F) in supranuclear vacuoles (indicated by S in B and C). E and F show particles in epithelial cells (marked E), and no particles in the underlying cells. The intestinal lumen is indicated by L in A. Scale bars 5 μ m (A, E and F), 1 μ m (B), and 500 nm (C and D).

4.2 The zebrafish model system; oral intubation with carboxylated latex particles

4.2.1 Uptake of 0.5µm carboxylated latex particles

To test the uptake of nano/microparticles in the zebrafish gut, we used carboxylated latex particles (CLPs). These particles are of uniform in size, are not degraded in the intestine or intracellular after uptake by phagocytic cells, and their strong fluorescence make them easily detectable by confocal laser microscopy. Samples of the intestine were dissected out 5min, 1h and 3h after oral intubation (n=3 in each time point) (fig.8).

Fluorescent 4E8 labelled sections revealed that CLPs attached to the mucosal layer and are taken up by epithelial cells in the anterior and second segment as early as 5 min after intubation (fig.8: A, B). No CLPs were seen in the posterior segment after 5 min (fig.8: C). After 1h and 3h CLPs were detected in both the brush border, epithelial cells and in the lamina propria (fig.8: D-I). CLPs are also seen in the muscularis/serosa after 1h and 3h, where blood vessels are located (fig.8: red arrows H and I). CLPs were more often observed inside epithelial cells of the second segment compared to other segments, however. no quantitative analysis was performed.

Anti-L-plastin staining showed CLPs overlap with positively stained leukocytes in the LP 1h after intubation (fig.9: D-I, K, L, red arrows). 5 min after intubation CLPs are seen attached to the mucosal surface or inside epithelial cells, they are not detected underneath the epithelium (fig.9: A-C, J).

Occasionally, accumulations of CLPs were detected inside single cells in the epithelium. (fig.10). This accumulation was only seen in the epithelium of the second segment, but it was always one single cells and never clusters of cells. The cell type was not determined.

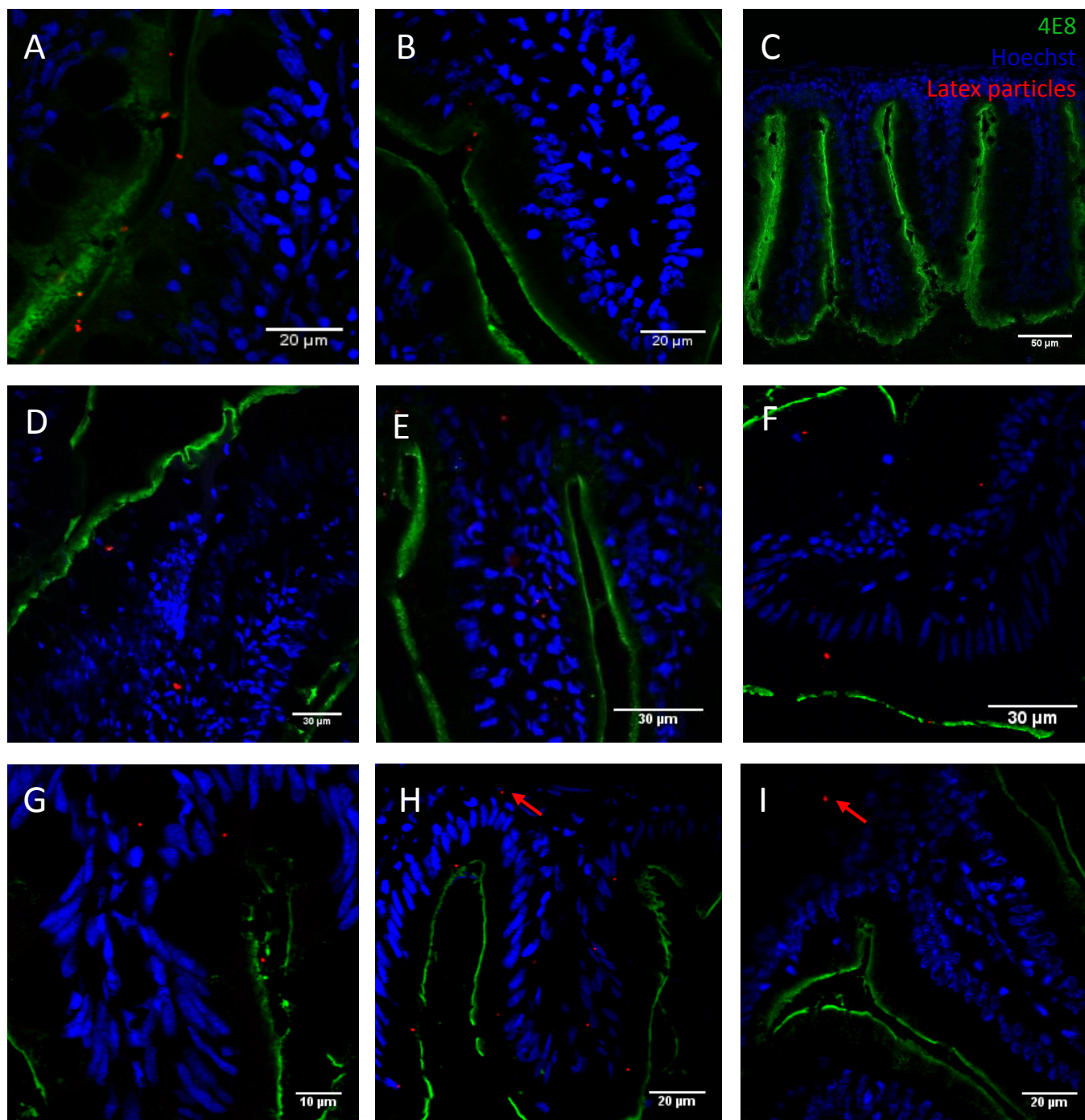


Figure 8: Uptake 0.5 μ m CLPs in the zebrafish intestine. Intestine isolated 5min (A,B,C), 1h (D, E, F), and 3h (G, H, I) after intubation. Anterior (A, D, G), second (B, E, H), and posterior segment (C, F, I). CLPs seen in red, brush border stained with 4E8 (green). After 5min, CLPs are seen in the brush border and epithelia of the anterior and second segments (A and B). After 1h and 3h CLPs are seen in the brush border, epithelia and sub-epithelial layers (D-I). Arrows in H and I indicate CLPs in the serosa.

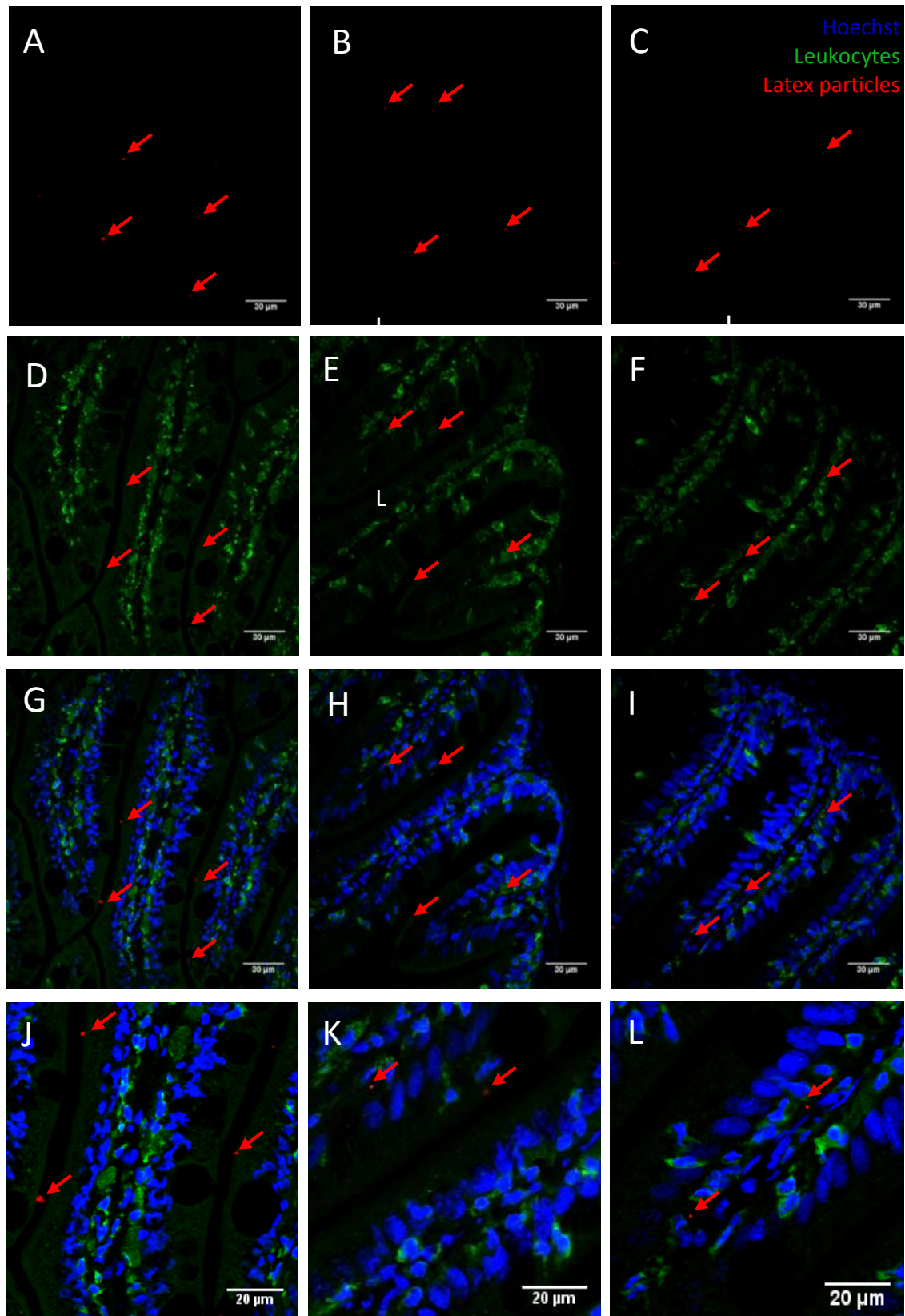


Figure 9: Uptake of 0.5µm, CLPs in the intestine, stained with anti-L-plastin antibody. The intestine is isolated after 5min (A, D, G and J) and 1h (B, C, E, F, H, I, K and L) after intubation. A, B and C show the CPL channel. D, E and F show the anti-L-plastin channel. J, K and L are magnification of G, H and I, respectively. Red arrows indicate particles and the intestinal lumen is marked with L.

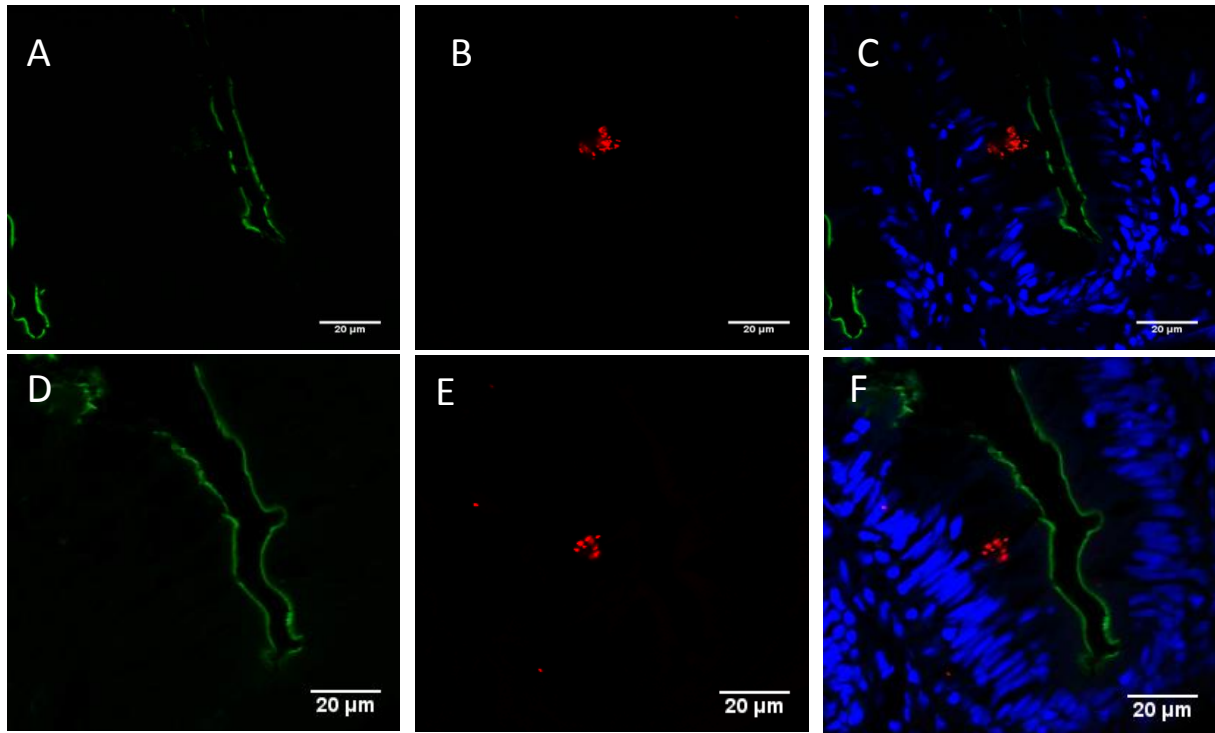


Figure 10: Two section of the zebrafish intestine second segment, 5h after oral intubation. A and D show 4E8 antibody, B and E show CLPs, and C and F show merged channels with nuclear counterstain. The two sections are serial sections.

4.2.2 Transport of 0.5µm latex particles to the spleen

After having established that CLPs are taken up in the zebrafish intestine, we investigated if CLPs are transported to other organs, especially with important functions in the immune system. For that purpose, we isolated the spleen, which one of the major lymphoid organs in teleost fish [96]. 1.5h after oral intubation, a distinct accumulation of CLPs were seen in the withe pulp region of the spleen (fig.11). Correlative microscopy and histochemistry with HE staining was used to identify the morphology the CLP containing tissue (fig.11). The white pulp are characterized as less dense areas in HE stained splenic tissue (fig.11: A, B, D and E, white squares). In samples 3h after intubation, we see that CLPs in the spleen are overlapping with L-plastin positive leukocytes in the white pulp, indicating that they were taken up by phagocytic immune cells (fig.12).

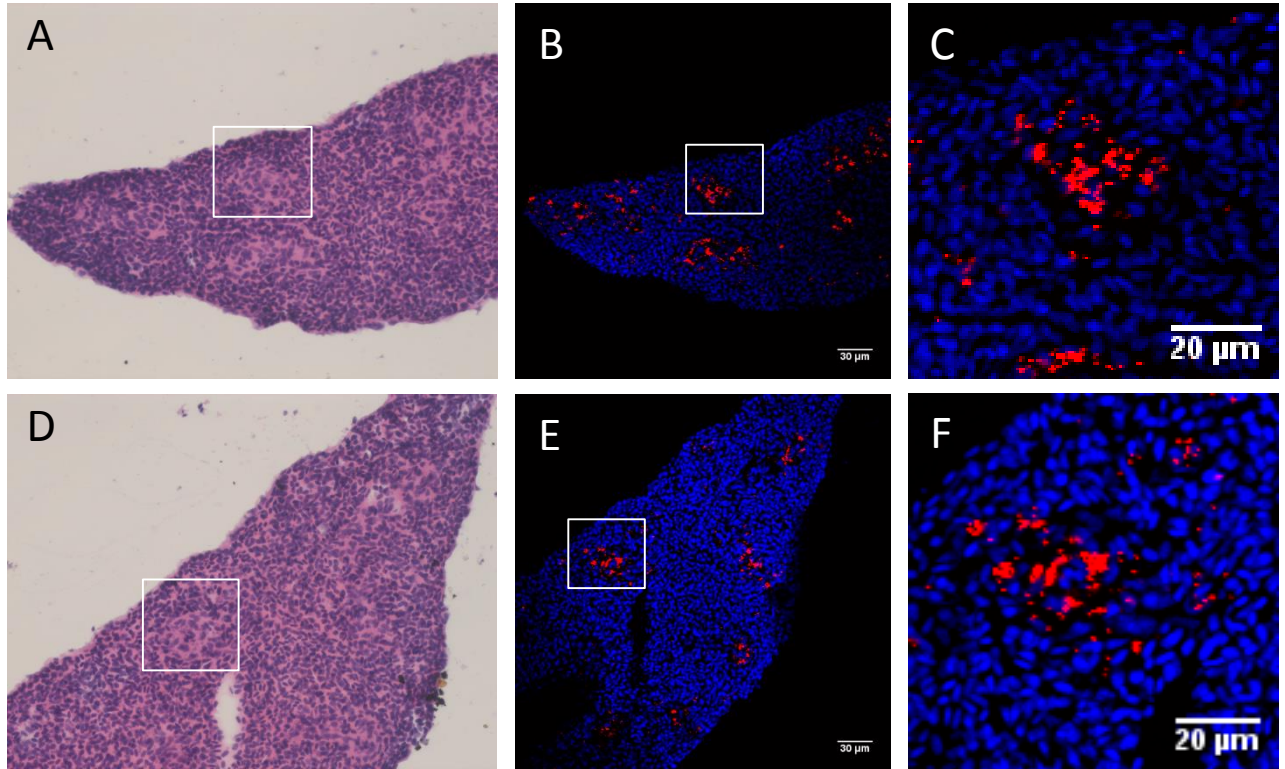


Figure 11: Zebrafish spleen 1.5h after oral intubation with 0.5 μ m latex particles. The same two section are stained with HE (A,D) and nuclear counterstain (B,C,E,F). C and F are magnification of particles in the white pulp of the spleen in B and E, respectively. The same area marked with white squares in A and B in C, and D and E for F. The white pulp can be seen as lighter areas in HE stained sections seen in the white squares in A and D.

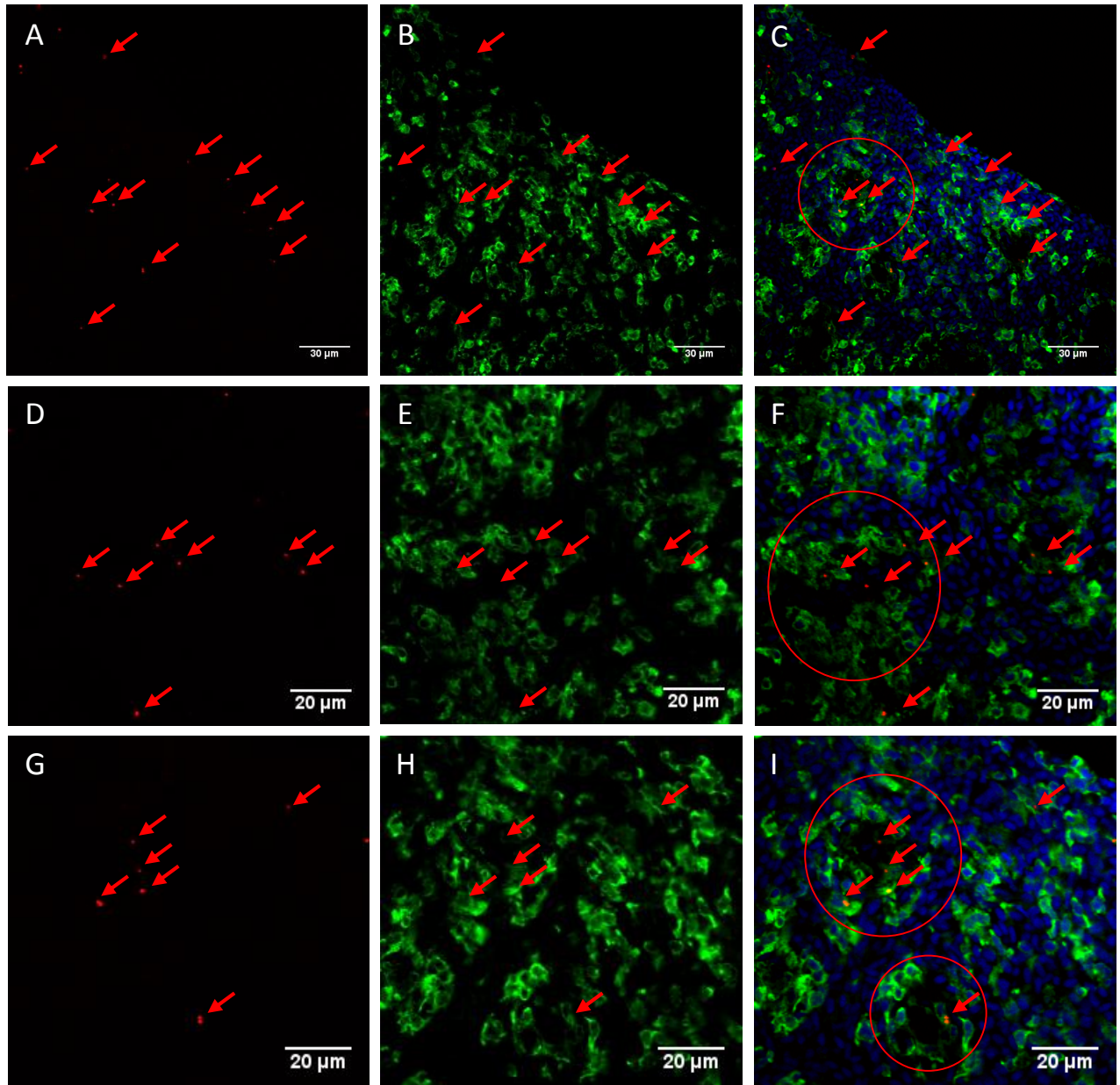


Figure 12: Zebrafish spleen 3h after intubation with 0.5µm CLPs. Sections are stained with anti-L-plastin and nuclear counterstain. A, D and G show the CLP (red) channel. B, E and H show the L-plastin (green) channel. C, F and I are merged channels with nuclear counter stain that show particles located in the white pulp around the central arterioles (red circles). The CLPs positions are marked with red arrows.

4.2.3 Uptake of 1 μ m carboxylated latex particles

To investigate the effect of particles size on uptake in the intestine of adult zebrafish, 1 μ m carboxylated latex particles were orally intubated. Like the smaller particles, 1 μ m CLPs were detected in the epithelium after 5 min and 1h (fig.13). However, the amount of particles in the epithelium and in the sub-epithelial layers was much less compared to 0.5 μ m, and no particles were detected in sections of the spleen and liver.

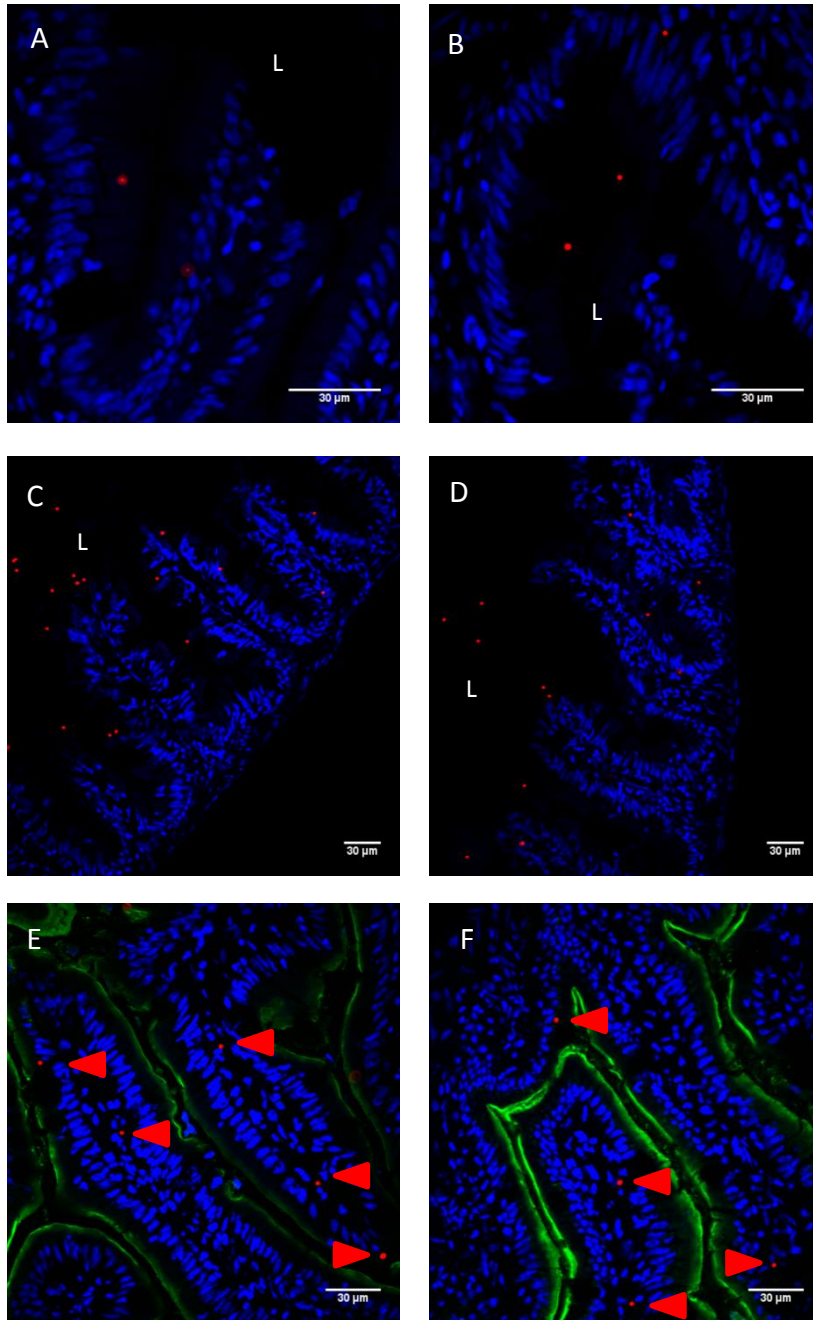


Figure 13: Zebrafish intestine injected with 1 μ m latex particles (red). Samples 5min (A, B, E, F) and 1h (C, D) after intubation, stained with nuclear stain (blue). E and F are stained with 4E8 (green) and red arrowheads indicate particles in the epithelial and sub-epithelial layers. The intestinal lumen is marked with L.

4.3 Uptake of Chitosan poly (I:C) nanoparticles

Chitosan poly (I:C) nanoparticles (Chi/pIC NPs) have been tested as an adjuvant in an injectable vaccine formulation against fish virus disease in adult zebrafish with promising results [92]. To investigate if these particles are suitable as vaccine carriers for oral administration of vaccines, we studied the uptake of fluorescently labelled Chi/pIC-NPs in this zebrafish model. Fluorescent Chi/pIC-NPs were prepared with chitosan conjugated with the far-red fluorescent fluochrom IRDye680® and injected into the zebrafish intestine by oral intubation. On sections stained with antibody 4E8, Chi/pIC-NPs were seen as a fluorescent cloud in the intestinal lumen 5 min after intubation, but were hardly detectable in the intestinal lumen 1h after intubation (fig.14). No Chi/pIC-NPs could be detected crossing the brush border or in the epithelium of the GI-tract at any time point. Furthermore, no Chi/pIC-NPs were observed in samples of the spleen and liver.

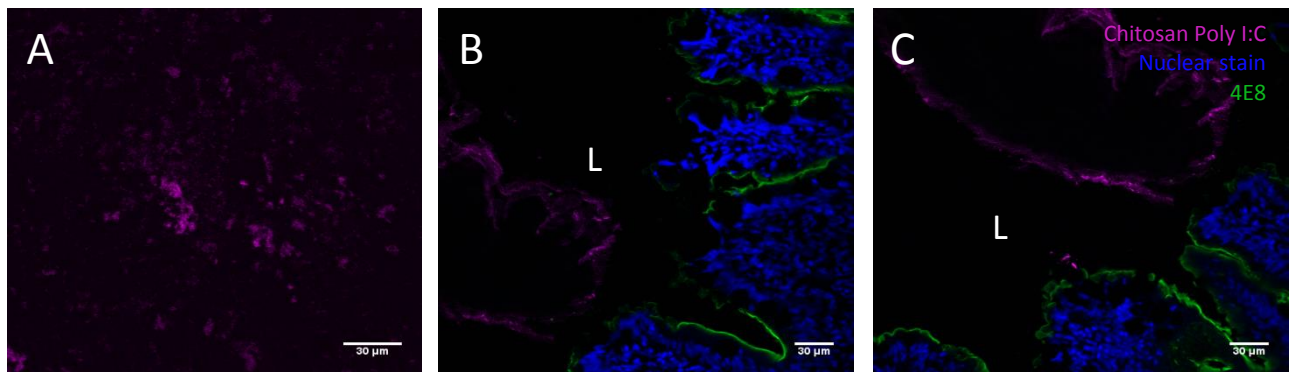


Figure 14: A show Chi/pIC NPs in solution. B and C are sections of the zebrafish intestine 5 min after oral intubation with Chi/pIC NPs (purple), sections stained with 4E8 (green) and intestinal lumen indicated with L.

4.4 Uptake of PLGA nanoparticles

PLGA NPs encapsulating anti-tuberculosis drugs have been developed in our group and tested both in mycobacteria-infected murine macrophages *in vitro* and in the zebrafish model for TB. PLGA particles injected into the blood stream of zebrafish infected with the close *M. tuberculosis* relative *M.m*, were shown to be rapidly taken up by macrophages and transported to sites of *M.m* infection [81]. Encapsulation of the efflux-pump inhibitor thioridazine in PLGA particles, used as adjuvant for antibiotic treatment against TB, decreased toxicity and increased killing of bacteria in zebrafish [97].

PLGA NPs are also tested as vaccine carriers in fish, both injected and orally delivered [61, 62]. Here we wanted to test how suitable PLGA NPs are as drug carriers for oral administration by using our zebrafish model. PLGA NPs encapsulating the red fluorescent dye rhodamine were orally intubated into the zebrafish intestine and samples prepared for cryostat sectioning. Already after 5 min, PLGA NPs were strongly localized in the Lamina propria (LP) and the epithelium of the intestine (fig.15: A and B). A large amount of particles can be seen around the nucleus of cells in the LP, the site where most intestinal leukocytes reside. This strong uptake is seen in both the anterior and mid sections of the intestine, and no particles are seen in the posterior section after 5 min. In samples 3h after intubation we do not see the same accumulation in the LP, however there are still many PLGA particles in and under epithelia cells, and some overlap with leukocytes in the LP (fig.15: C-F). We also find particles in the spleen 3h after oral intubation, with the same accumulation around the white pulp as seen in the intestine LP 5 min after oral intubation (fig.15: G-H).

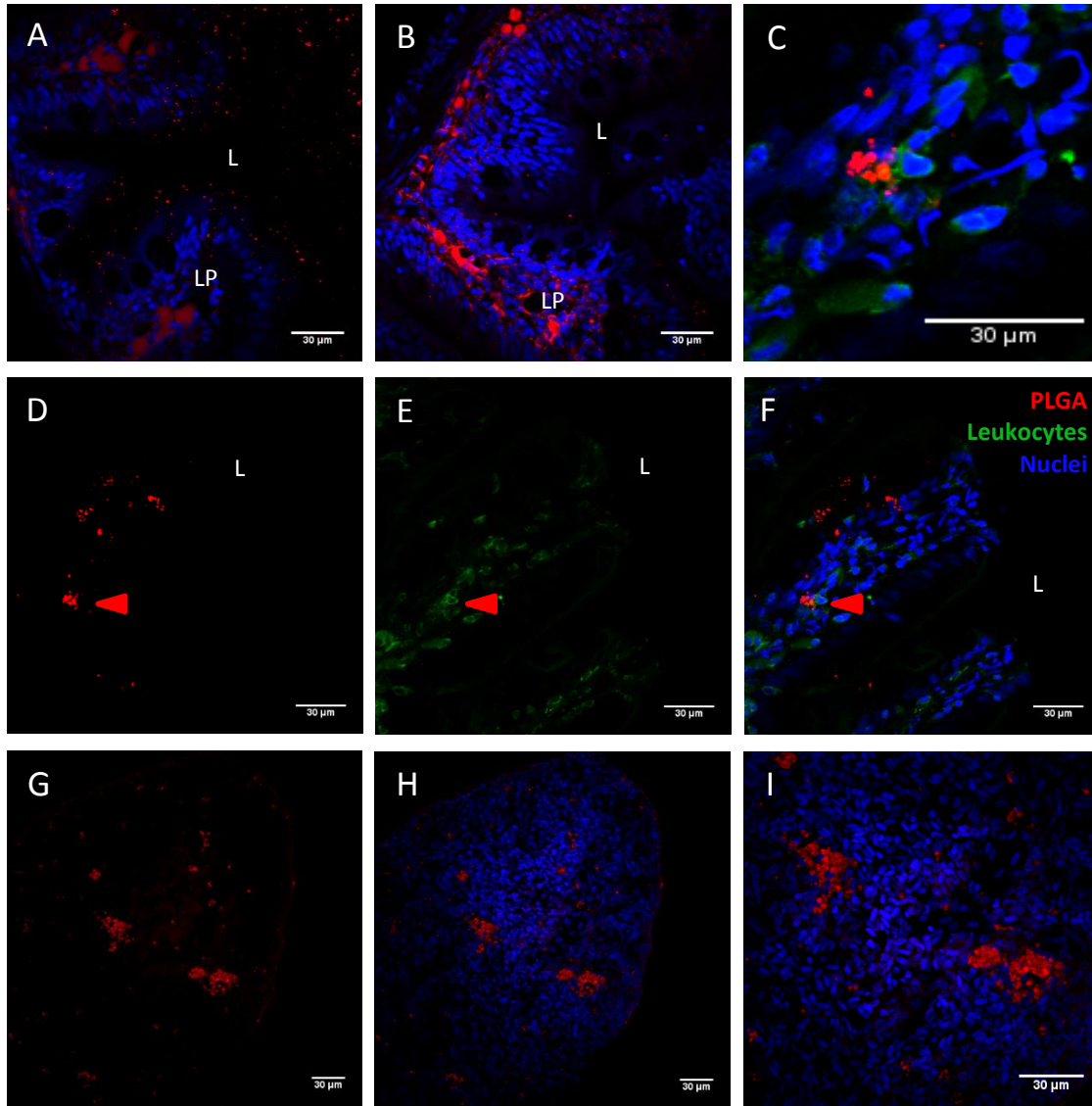


Figure 15: A and B show Zebrafish intestine 5 min after intubation with PLGA particles (red), anterior and second segment, respectively, stained with nuclear stain. C is a magnification of sections of the second segment 3h after intubation (area marked with a red arrowhead in D-F), stained with nuclear stain and anti-L-plastin antibody. D and E show the separate PLGA and anti-L-plastin channels, respectively. F show the channels merged with nuclear counterstain. G-I show the spleen 3h after oral intubation with PLGA particles. G show the PLGA (red) channel alone and H show the same channel merged with the channel for nuclear stain. I show a magnification of the central arterioles with red PLGA particles. The intestinal lumen is marked with L.

4.5 *Mycobacterium marinum* infection

The fish pathogen *M. m* is thought to have a natural infection route through the intestine [83]. We wanted to utilize our zebrafish model to look closer at *M. m* infection over the mucosal epithelium in the intestine. To do this, live red fluorescent bacteria were injected into the zebrafish intestine and samples of the intestine, spleen and liver were investigated. In samples 5min after intubation, bacteria were only seen in the intestinal lumen (fig.16 K). After 1h, bacteria were detected in and around leukocytes in the intestinal serosa (fig.16: A-C and L). After 5h, samples of the spleen show aggregates of bacteria that overlap with leukocytes in what is most likely the white pulp (fig.16: D-F and J). In addition, intestine samples after 5h, we see large amount of bacteria co-localizing with leukocytes in liver tissue directly adjacent to the intestine (fig.16: G-I). However, in samples only taken of the liver no bacteria were detected.

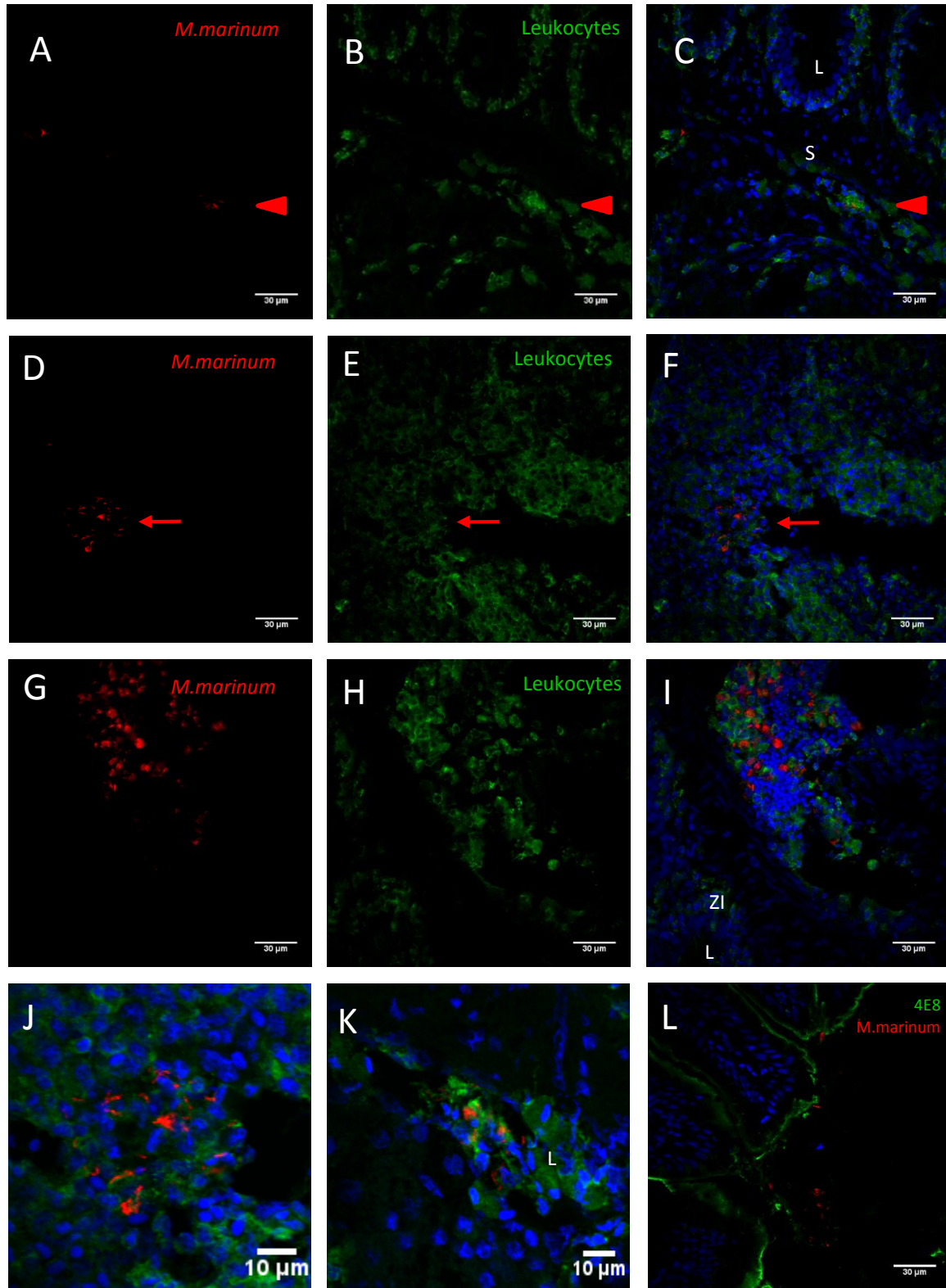


Figure 16: A-C are Samples of the zebrafish intestine 1h after oral intubation with *M.m*, stained with anti-L-plastin. Bacteria are seen around the serosa (S), and K show a magnification of the bacteria (area marked with a red arrowhead in A-C). A and B show the separate bacteria and the L-plastin channels, respectively. D-F are the zebrafish spleen 5h after oral intubation where accumulation of bacteria are seen (red), D and E show the separate bacteria and anti-L-plastin channels, respectively. J shows a magnification of the area with bacteria (area marked with red arrow in D-F). G-I show zebrafish intestine and liver 5h after oral intubation. Liver tissue is seen adjacent to the intestine (marked ZI) with a large number of bacteria and leukocytes. G and H show the bacteria and the L-plastin channels, respectively. L show red fluorescent bacteria in the intestinal lumen 5min after intubation, stained with 4E8 (green). Intestinal lumen marked L.

5 Discussion

In this thesis, I have used a zebrafish model system to carry out a series of experiments aimed to study antigen uptake and oral administration of potential vaccine carriers in the teleost fish intestine. First, I investigated the presence of antigen sampling cells in the zebrafish intestine and compared my findings to results from other teleost species, in particular in salmonids. Second, I established the zebrafish as a model for uptake of nano/microparticles by using fluorescent, non-degradable carboxylated latex particles, and show that the particles can be traced in different tissues after they have crossed the epithelial layer. Furthermore, I characterized the uptake of particles already established as drug carriers. Third, I use this model to show how the fish pathogen *M.marinum* can infect by crossing the epithelia of the zebrafish intestine.

5.1 Morphology of the zebrafish I tract

The morphology of the zebrafish intestine is often used as a model for the intestine in other species, as the embryological development is very well characterized [98]. Teleost intestinal architecture differs from the mammalian system, as it is simpler with fewer intestinal layers. The teleost intestine starts with the epithelium bordering the intestinal lumen and the underlying lamina propria, which together creates intestinal folds. Under the intestinal folds, a layer of circular and smooth muscles (muscularis) and the serosa create the base of the layers. Although the zebrafish intestine differs from other teleost species like salmonids, as it does not have an acidic stomach [99], the structuring of the intestinal layers is the same in zebrafish [75]. We therefore reasoned that the zebrafish is a good model for antigen uptake in the intestine that may make it possible to transfer our studies to other species.

Therefore, we first wanted to characterize the zebrafish intestinal morphology, including intestinal folds, epithelial layer with different cells types, lamina propria, muscularis and serosa. This allows us to relate particle uptake to the morphological structures.

To visualize the anatomy of the intestine and other tissue samples we used two different preparation methods, paraffin and cryostat sectioning. Paraffin sectioning is useful for preserving morphological structures of tissues and therefore used for classical hematoxylin and eosin (HE) staining. However, harsh solvents like xylene are used and the method can

mask antigens, making it necessary to add an antigen retrieval step to the protocol. Most importantly, this method is generally not compatible with fluorescent dyes, as their fluorescence are often lost during preparation of the samples [100]. I therefore used cryostat sections for samples containing fluorescently labelled material, which is a gentler and less time-consuming method. Cryostat sections were also used for UEA-lectin staining of antigen sampling cells in the salmonid intestine (Fuglem et al.)

The general morphology of the zebrafish intestine was visualized by HE staining on paraffin sections. There are differences in morphology and cell type composition between the intestinal segments, which has an indication for the different functions of the segments. In the anterior segment, there are mostly enterocytes, an indication mainly of nutrient uptake. In the posterior part of the second segment, specialized enterocytes with supranuclear vacuoles (SV) are abundant, which may be involved in antigen sampling from the intestinal lumen. In the posterior segment, the intestinal folds are shorter and in a longitudinal direction, compared to being transverse in the anterior and second segment, giving the segment a structure more like the mammalian colon.

5.2 Identification of antigen sampling cells

In mammals, antigens are sampled from the intestinal lumen and presented to immune cells by M cells [94]. So far, no equivalent to M cells have been identified in fish, but there is evidence of specialized enterocytes or so-called antigen sampling cells in the teleost intestine [12, 31, 37].

In zebrafish and other teleost species, antigen sampling cells are claimed to be specialized enterocytes with large SV where macromolecules are taken up [12, 31, 32]. These distinct structures are clearly visible in HE sections in the posterior segment of the zebrafish intestine, and are the site where we expected particles to be taken up. Fuglem et al. showed that in salmonids, antigen sampling cells stain positive for UEA-I- and negative for WGA- lectin [37]. M-cells in the mouse intestine are characterized by the same lectin staining pattern, which let the authors to conclude that these cells in the salmonid intestine resemble mammalian M-cells (based on the lectin-staining pattern, uptake of particular antigen and morphology). The same staining protocol was here tested in zebrafish, but in contrast to mouse and salmon samples, no staining along the whole intestine was visible for UEA-I

lectin. This was especially carefully checked in the second segment of the intestine, where we expected cells to be UEA-I positive. Lectins bind to specific sugars in carbohydrate residuals [39], and this specific carbohydrate-labelling pattern is evidently evolutionary conserved in mammals and salmonids, but not zebrafish [40]. The lack of UEA-I lectin binding and the fact that we did not find cells with irregular microvilli argues that zebrafish do not have M cell-like cells, like salmonids. This may mean that zebrafish have lost this cell type over time, even though zebrafish branch off earlier than the salmonids from the evolutionary tree [9]. This result may also indicate that SV found in zebrafish and other fish species may not be related to M cell-like cells found in the salmonid intestine.

Furthermore, mammalian M-cells are characterized by their ability to take up whole bacteria or even several μm -sized yeast [101]. In salmon, M cell-like cells are able to rapidly take up 10nm BSA-gold particles. The original experiments by Fuglem et al. was done *ex vivo*, where samples of salmon intestine were submerged in a solution containing gold particles for two minutes. They found single, or clusters of enterocytes in the second segment containing accumulated gold particles. To repeat this experiment in zebrafish, 10 nm BSA-gold particles were injected into the GI-tract by oral intubation. Accumulations of gold-particles were detected in almost all cells in the second segment, except in cells near the bottom of the intestinal fold. However, the main difference between the experiments in our and the Fuglem et al. studies, beside the *ex vivo* versus *in vivo* exposure, is that the gut epithelia in the zebrafish in our study was exposed to gold-particles for a longer period (1h).

This long exposure to particles in the intestine, 1h compared to 2 min in Fuglem et al., may have caused masking of cells rapidly taking up particles, as all cells in the posterior segment may eventually take up the small particles with the prolonged exposure. However, the pattern of uptake, with little or no uptake near the bottom of the intestinal fold, matches with the placement of supranuclear vacuoles seen in the HE stained sections. We also saw that many of the gold particles in enterocytes were inside compartments that resemble supranuclear vacuoles.

In salmonids, uptake of gold particles was furthermore detected in cells underneath the epithelium with 'arms' extending into the intestinal lumen. These cells displayed a dendritic cell-like morphology. Neither these DC-like cells, nor particles in cells of the sub-epithelial layers were seen in our zebrafish samples. However, it needs to be mentioned that only the posterior part of the second segment was investigated by EM in this study. Whether other cell

types, in different segments of the intestine, take up BSA-gold particles or the particles are taken up by all cells throughout the whole intestine needs to be further investigated.

5.3 Uptake of carboxylated latex particles (CLPs)

Little is known about the uptake of larger particles in the teleost intestine. Several studies have reported that particles up to 3µm are taken up in the intestine of different teleost species, with the uptake detected mainly with indirect methods [102, 103]. However, the uptake of larger particles by the intestinal epithelia has not yet been characterized in detail. To address this open question, we used CLPs, since they are uniform in size and are most importantly not degraded in the intestinal lumen or intracellularly in cells. CLPs are routinely used to study phagocytosis in Mφs and DCs, which can effectively phagocytose particles in the range between 200nm and 1µm [104]. To study if the size of the particles has an eventual effect on uptake, we used 0.5µm and 1µm fluorescent CLPs for oral intubation experiments.

Directly after oral intubation large amounts of CLPs of both sizes were found in the intestinal lumen. However, there was a clear difference in uptake of particles in the intestinal epithelium between the two CLP sizes. The smaller 0.5µm CLPs were seen more frequently inside the epithelia or in the sub-epithelial layers, compared to the bigger particles. In addition, 0.5µm CLPs were found to accumulate in the spleen already after 1.5h, whereas 1µm CLPs were not detected in spleen samples even after 5h. Different cellular mechanisms involved in the uptake of 0.5µm vs. 1µm CLPs may account for the size-dependent uptake, like phagocytosis and endocytosis, seen in our experiments. However, the fate of the particles after uptake may be different. Even though 1µm particles are seen to a less extent in the epithelia, they may be transported across to other tissues than the spleen or liver. This has not been addressed in this study and has to be determined in future experiments. Alternatively, 1µm CLPs might not leave the intestinal epithelia after uptake, which would mean that they are taken up very poorly, as even after 5 hours no accumulation of CLPs are seen in the epithelia. Interestingly, a previous study in salmon found that the optimal particle size for uptake in the intestine was 1µm [103]; which resulted in higher accumulation of 1µm particles in the spleen compared to 0.5µm particles .

Using tissue sections of the intestine, we can only see a ‘snapshot’ of the particle uptake at the time-point the samples were taken and frozen down. This method is therefore less suitable for quantitative analysis of CLP uptake. To quantify the uptake of particles in the spleen, a flow cytometry protocol was set up (Supplementary data). This method is well suited for quantitative analysis, as it is a laser-based technology that detects and counts single cells that are fluorescently marked. In this case, it will count cells that have taken up CLPs. This makes it especially suitable for CLPs uptake studies, as they are very brightly fluorescent. However, the cell count from spleen samples of fish intubated with 0.5µm or 1µm CLPs did not differ from the negative control. Since it was already established that 0.5µm CLPs accumulate in the spleen after 1.5h, we assume that the lack of detection of CLP positive cells are caused by technical procedure, rather than representing actual results. Further optimization of the protocol is therefore necessary.

After we established that 0.5µm CLPs were taken up across the epithelia, I continued working with this particle size. As the literature on zebrafish points out the posterior part of the second segment as especially important in the immunological response, and as the site of antigen sampling cells with supranuclear vacuoles [32, 74, 75], we wanted to see if CLPs were more actively taken up in this area of the intestine. Samples from several individuals (n=3) at different time-points after oral intubation indicate a slightly higher uptake of particles in the second segment. Nevertheless, due to reasons mentioned above, quantitative analyses to confirm this, was difficult to perform.

Occasionally, single cells in the epithelium containing larger accumulations of particles were seen. These cells were sparse, but were exclusively located in the second segment, indicating that CLPs accumulated inside SV of antigen sampling cells. However, why these CLP accumulations in single cells are so sparse and not found along the whole epithelia, as SV are evident in most epithelia cells in the posterior part of the second segment is not clear. The identity of the cell type containing these accumulations can eventually be answered by the use of correlative confocal and light microscopy.

We also do not know how the CLPs cross over the epithelia. In mammals, normal enterocytes are known to taken up antigens, which can result in degradation, translocation across the epithelia, or antigen presentation to underlying T cells [105]. However, these processes are not well documented in the fish intestine. Particles can also be taken up by intraepithelial leukocytes, like DCs, as was reported by Fuglem et al.

In zebrafish, we observed CLPs that overlap with anti-L-plastin positive cells both in the sub-epithelial layer of the intestine and in the spleen, 1h and 3h after intubation. This argues strongly for that particles are transported from the intestine to the spleen via leukocytes and not freely in the blood. This is also supported by studies in zebrafish embryos, where particles, directly injected into the blood circulatory system, are quickly removed from the bloodstream by Mφs along the endothelia within minutes [81]. We have also not detected any leukocytes with projections protruding into the intestinal lumen, either with confocal or by electron microscopy. For our confocal microscopy analysis, the leukocytes were labelled with anti-L-plastin; however, this marker does not label the whole cell, which therefore makes it difficult to see potential ‘arms’ projecting into the intestinal lumen.

5.4 Chitosan poly I:C particles

Chitosan has been tested as adjuvant and drug carrier in a number of studies both in mammals and in fish [52, 56, 91]. Combining chitosan and with the TLR3 agonist poly (I:C) produces particles with many advantages such as protection of poly (I:C) and encapsulated vaccine antigen from degradation, enhanced uptake by APCs (signal 2 facilitator adjuvant), slow release and targeting the mucosa due to their muco-adhesive properties [53, 91]. Collectively, these data make these particles a good candidate to test in the zebrafish model for particle uptake in the intestine. Since these particles were used successfully as adjuvants against VHS-virus infection in zebrafish [92], we were interested to see if the same particles were suitable for oral administration.

Results from the oral intubation experiments with fluorescently labelled particles show that the particles reach the intestinal lumen. However, no fluorescent signal could be detected in the epithelial layer or in spleen and liver samples. The particles in the lumen also appeared to have partly dissolved, and appeared like a dust-cloud in the intestinal lumen. I therefore tentatively conclude that these particles are not suitable for oral administration in this form. However, chitosan is a very versatile compound and optimization of the particle preparation may aid the development of chitosan particles, which are more suitable for oral application.

5.5 PLGA particles

PLGA particles have been tested as a vaccine carrier in teleost fish with promising results [61, 62]. PLGA particles encapsulating anti-mycobacterial drugs have shown to effectively treat mycobacterial infections when injected in the zebrafish embryo model for TB by our group [81, 97]. We therefore wanted to investigate if PLGA particles were also suitable for oral administration. The results show that the particles, as CLPs, are taken up in high numbers, rapidly after intubation, and they co-localized with leukocytes in the lamina propria.

We also observed a surprisingly strong uptake of particles after 5 min across the epithelia, as there are cells heavily stained in the lamina propria, which was not seen with CLPs. One explanation for this difference could be that PLGA particles are taken up into M ϕ s and as they start to degrade, their enclosed dye leaks out into the leukocyte cytoplasm. We also see this phenomenon in the spleen, where there were cells that were completely stained red after 2h, and in contrast to the CLPs no single particles of PLGA were evident. However, in sections of the intestine, 1h and 3h after intubation, we mainly saw single red particles comparable to CLPs. The significance of these observations is not clear at this time.

As particles are detected in the spleen after oral intubation, we can argue that they were transported away from the intestine to major organs of the immune system. Taken together, the results imply that PLGA particles are a promising approach for encapsulation of oral vaccines.

5.6 Infection of *Mycobacterium marinum* via the intestine

A previous study by Harriff et al. [83] established that *M.m* could infect different organs across the zebrafish intestine, by assaying granuloma formation and colony forming units from tissue samples. This strongly argues that the natural route of infection of *M.m* is via the intestine. Here, we wanted to use the zebrafish model to study the route of infection, so to understand how pathogens can infect via the gut. Which may prove to be important information for developing suitable oral vaccines. We show that the bacteria have crossed the mucosal barrier within 1h, and after 5h we found accumulation of leukocytes with intracellular bacteria in the spleen and liver. *M.m* is primarily taken up by M ϕ s, and is able to survive intracellularly in these cells. As infected M ϕ s are not able to kill intracellular *M.m*,

infection is contained by the formation of granulomas [77]. In all tissue samples where we found M.m after intubation, bacteria overlapped with leukocytes. This makes it likely that bacteria are phagocytosed by macrophages after crossing the epithelia, and are transported inside these cells to the spleen and liver, and likely other tissues. The bacteria were seen overlapping with leukocytes already after 1h, and migration of these cells may explain why granulomas are found in many different tissues, as they are first described at 3 days post infection [106].

This opens the question of how M.m is able to cross the epithelium and to be taken up by Mφs. This bacterium is about 1-2μm in size, e.g. the same size or larger than the 1μm CLPs, which are taken up to a less degree as 0.5μm particles. However, in contrast to the CLPs, M.m has several components in its cell wall (lipoglycans; lipoarabinomannans and lipomannan), which activate PAMP-receptors such as TLR2 and TLR4 [107]. It is therefore likely that the uptake of M.m is an active, receptor mediated uptake, and probably differ from the mechanisms involved in the uptake of 0.5μm CLPs.

6 Conclusions

In this thesis,

(1) Zebrafish was established as a model system for testing nano/microparticles as potential vaccine carriers for teleost fish oral vaccination. This was demonstrated using carboxylated latex particles (CLPs) for oral intubation. CLPs were observed in the intestinal brush border and the epithelial layer 5 min after oral intubation. Following 1h and 3h exposure post intubation, CLPs were additionally identified in the sub-epithelial layers, in association with leukocytes. CLPs were also transported into other organs, as accumulation of particles were seen in the splenic white pulp following 1.5h after oral intubation. In addition, this study provides an indication on the importance of optimal particle size for uptake. We observed that 0.5 μ m CLPs were more easily taken up than 1 μ m CLPs, and was the only particle size detectable in the spleen. The model was further tested with two possible vaccine carriers, encapsulating fluorescent dyes. Demonstrating that the Chitosan poly (I:C) particles were not suited for oral delivery and PLGA particles were shown to be taken up across the epithelia.

(2) Further, pathogen infection through the intestine was investigated. *Mycobacterium marinum* were seen across the intestinal epithelia and the bacteria were transported into other organs within hours. At such locations, they can replicate within macrophages and induce a granulomatous inflammation. In addition, leukocyte staining indicated that the bacteria were most likely transported from the gut into other tissues within macrophages.

In addition, the results in this thesis indicate that the zebrafish do not have M cell -like cells as salmonids have, as both the UEA-I staining was negative and we could not detect an irregular microvilli morphology known to be associated with M cells. However, identification of supranuclear vacuoles and the indication of a larger uptake of latex particles in the second segment of the intestine, gives further reason to believe that the second segment is of immunological importance, as previously reported [1, 75].

Finally, although the long-term application of the analysis carried out in this thesis has been undertaken with an eye on commercial fish, such as salmon, the zebrafish model used here allows a spectrum of experiments to be carried out relatively quickly and efficiently that would be much more difficult, expensive, and time-consuming in other species.

7 Future perspective

7.1.1 Immune cells and uptake of particles

There is still more work to be done with identification of antigen sampling cells, in zebrafish and other teleost fish. Even though I see more particles taken up across the epithelia in the second segment of the intestine, this is not quantified. Other studies also identify the second segment of the intestine as immunologically important and claim enterocytes with supranuclear vacuoles to be antigen sampling cells. However, there is no evidence that these cells are connected to the immune system and antigen is transferred to antigen-presenting cells, like Mφs and DCs. Therefore, more research is needed on antigen sampling cells and underlying leukocytes. In addition, it would be interesting to see if zebrafish leukocytes are able to protrude arms into the intestinal lumen, or if the whole cell can enter the lumen and sample for antigens, as is indicated in salmon [37, 101]. Moreover, to identify the optimal size for uptake in the zebrafish intestine, particles smaller than 5.0 μm should be tested for uptake. Further, correlative microscopy with confocal laser and light microscopy, may aid in identification of morphology and cell types involved in uptake of particles/antigen.

7.1.2 Analysis of the immune response

To create a successful vaccine formulation for oral administration, the vaccine needs to be protected in the intestinal environment, and as much as possible of the vaccine needs to be taken across the epithelium and presented to immune cells. In addition, an appropriate immune response with immunological memory against the desired pathogens needs to be achieved. Here we present a model that can test the vaccine formulation for survival in the intestinal environment and for crossing the intestinal epithelia. To complete the model, one also needs to quantify the uptake and compare the immune response to the different formulations. Even though the flow cytometry experiment done on zebrafish spleen was negative, I believe the method would be good for quantitative analysis of the uptake, as you can look at uptake in the intestinal tissue, spleen, liver, kidney, and even blood samples [108]. To look at the immune response, expression of inflammatory cytokines (TNF-α, INF-γ, IL-1β), iNOS and other inflammation markers can be measured over time by qPCR. In addition, better markers for immune cells, for distinguishing between innate (DCs and Mφs) and adaptive (B- and T- cells) immunity will give a good overview of the immune response. This

is especially important when testing encapsulating compounds, like chitosan, which acts as a signal 2 facilitator adjuvant.

7.1.3 Mycobacteria marinum infection

In this thesis, we show that M.m can infect through the zebrafish intestine. However, there little is known about how the bacteria pass the mucosal barrier. Is the uptake of the bacteria active or passive? Does it pass through the epithelial cells or between the gap junctions?

Understanding the rout of infection of a pathogen and knowing how it passes the immune system at the mucosal site may aid in the development of drugs against TB.

8 Appendix

8.1 Supplementary methods

8.1.1 Flow Cytometry

Flow cytometry is a technology used to analyze fluorescently labeled particles in fluid. This protocol for flow cytometry is based on [98].

The spleen was dissected out from fish that were sacrificed 1h and 3h after intubation with 0.5µm or 1µm red fluorescent carboxylated latex particles, as used in previous intubation experiments. The number of latex particles in suspension was calculated from the formula described above, and the 1µm and 0.5µm particle suspension were diluted in PBS so that the number of particle in both solutions were approximately the same. For each group analyzed, three fish were sacrificed (n=3). One group was intubated with PBS as a control.

After the fish were sacrificed, the individual spleens were dissected out and disintegrated through a 70µm cell strainer (BD Falcon™) and washed twice in PBS 2% fetal bovine serum (FBS), centrifuged at 600g for 7min and re-suspended in PBS 2% FBS. In each group, spleens from three fish were pooled after disintegration to ensure a sufficient number of cells for flow cytometry analysis. Before analysis, the cell suspension was filtered through a 40µm Cell Strainer Cap. The samples were analyzed on a Gallios™ Flow Cytometer - Beckman Coulter, at NMBU. The samples of the PBS control was analyzed first to compensate for background fluorescence.

8.2 Supplementary results

8.2.1 *Francisella noatunensis ssp. orientalis*

Francisella species are anaerobic, gram-negative, intracellular bacteria. They are found in many different environments and causes diseases in a wide range of hosts, including humans and fish. The strain *F.tularensis* is a highly infectious, intracellular pathogen in humans that

some worry can be used in bioterrorism [99]. A number of fish species, are prone to infection, including cod, tilapia and Atlantic salmon. Francisella infection in fish develops in a similar way in all host species, and the infection is commonly characterized by the presence of granuloma and high morbidity [100, 101].

Francisella noatunensis ssp. Orientalis (F.n.o) live in warmer environments and have an optimal growth temperature at 28 °C. The bacteria infect fish like zebrafish and tilapia, and cause granulomatous disease. A zebrafish model to study the Francisella infection is already established [102]. However, the natural infection rout of the bacteria are still poorly understood.

Live, fluorescent *F.n.o* bacteria were orally intubated into the adult zebrafish intestine as done with *M.m*, and samples of the intestine and spleen were taken out after 5min, 1h and 3h. Unfortunately no fluorescent bacteria were detected in the intestinal lumen, epithelia or sub-epithelial layers in any of the samples.

8.2.2 Flow cytometry with 0.5µm and 1µm CLPs

Flow cytometry on pooled zebrafish spleen, 1h and 3h after oral intubation showed no difference in fluorescent signal compared to the control.

8.3 Supplementary recipes

8.3.1 7H9 liquid culture medium

Adaption from [67]. 4.7 g Difco Middelbrook 7H9 broth base dissolved in water, add 4 ml 50% (v/v) glycerol and brig up to a final volume of 900 ml, autoclave. Can be store up to 3 months at room temperature. Before use, add 100 ml ADC stock and 2.5 ml of 20% (v/v) Tween 80. Can be stored with ADC for up to 2 months.

8.3.2 ADC supplement

Adaption from [67]. ADC is a growth supplement that is essential for the growth of the slow-growing mycobacteria. Sodium chloride is there to ensure osmotic equilibrium, albumin binds toxic agents in the media and dextrose is an energy source for the bacteria.

Dissolve the following in 700 ml H₂O: 50 g BSA (fraction V, Sigma-Aldrich), 20 g dextrose and 8.5 g NaCl. Bring up to 1 liter final volume and filter the solution through a 0.22µm filter. Can store up to 6 months at 4 °C.

8.3.3 Tricaine stock solution

The following was adapted from [67]. For 100 ml, 4 mg/ml Tricaine stock solution, 400 mg Tricaine methansulfonate MS-222 (Argent laboratories group Inc. Redmond, WA, USA) was dissolved in 97.9 ml water and 2.1 ml 1M TrisCl (pH 9). The solution was adjusted to pH 7.4. For sedation of adult Zebrafish dilute the stock to 150 mg/L in systemic water.

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